

A COMPARATIVE STUDY OF THE GLOMUS COCCYGEUM AND THE CAROTID BODY

W. HENRY HOLLINSHEAD

*Department of Anatomy, Duke University School of Medicine,
Durham, North Carolina*

TWO PLATES (SEVEN FIGURES)

INTRODUCTION

The carotid body (glomus caroticum) and the glomus coccygeum were apparently first homologized by Luschka (1862), who regarded them as glands, and shortly thereafter by Arnold (1865). The latter interpreted them as glomeruli, believing the blood vessels were the important constituent of both, and that the cells represented thickened "skin" (endothelium) of the vascular wall. Thirty-seven years later Kohn ('02) again grouped these organs together, but, on the basis of their supposed morphology and embryology, included them as members of the chromaffin (paraganglionic) system.

The non-paraganglionic nature of the glomus coccygeum was shown a few years later by Stoerk ('07), and this body is now generally regarded as a group of arterio-venous anastomoses, the characteristic cells of which represent modified muscular elements (Schumacher, '08; Clara, '27; Masson, '37). More recently the carotid body has been shown to be a chemoreceptor, and thus also apparently removed from the chromaffin¹ group (see the reviews by Heymans and Bouckaert, '39; Gesell, '39; Schmidt and Comroe, '40; and Hollinshead, '40a). Since the beginning of this century, therefore, the

¹Goormaghtigh and Pannier ('39), among others, believe that there are some chromaffin cells in the carotid body; in common with all recent investigators, however, they report that the chromaffin reaction is not given by the majority of the cells.

coccygeal and carotid bodies have been once again reclassified (and apparently separated), the first primarily because of its embryology and morphology, the second primarily because of its physiology. There is, however, no experimental evidence as to the function of the glomus coccygeum, and there are at present pronounced differences of opinion regarding the histology and embryology of chemoreceptor tissue.²

Schumacher ('38) has again revived the old comparison between these structures, stating that the glomus coccygeum, the carotid body, and the supracardial and abdominal bodies, are morphologically indistinguishable. Each, according to his interpretation, represents a specialized development of the arterio-venous anastomosis. In view of the known function of the carotid body, this implies that the glomus coccygeum also is a chemoreceptor. Schmidt and Comroe (op. cit.) allude to it as morphologically similar to the carotid body, remarking that it has never been investigated for chemoreceptor function. Moreover, if the present histological interpretations of the glomus coccygeum are correct, Schumacher's comparison also suggests that chemoreceptor tissue, arising by specialization of arterio-venous anastomoses, may be much more widely distributed than is now recognized.

In the absence of opportunity for determining directly the function of the human glomus coccygeum, these supposed similarities can be investigated only by morphological means, as Schumacher himself has done. Nevertheless, his conclusions are of such potential importance to our concepts, both of the glomus coccygeum and of chemoreceptor tissue, that they can be accepted only after careful scanning of the evidence. Unfortunately, Schumacher has not revealed the details of the methods he used, nor of his observations. He merely states

² Besides the carotid body, this term, in accordance with present evidence, includes similar masses about the origin of the subclavian arteries (the aortic glomi of Nonidez); in relation to the ascending aorta and pulmonary artery (variously termed, collectively or individually, the supracardial paraganglion of Penitschka, supracardial bodies, glomus pulmonalis, aortic paraganglion or bodies, aortic arch bodies); and probably, in the mouse and rat, along the branches of the coeliac artery and nerve plexus ("abdominal vagal paraganglia" of Goormaghtigh).

that the bodies he observed seemed identical in cell type and in vascular arrangement.

The present work was undertaken to investigate once again these supposed similarities, comparing the carotid body, a typical chemoreceptor, with the glomus coccygeum. As an example of a somewhat less complex grouping of arterio-venous anastomoses, the glomeruli caudales from the cat were studied. The results are in agreement with the assertions of many other authors, including Schumacher himself, as to the similarities between the glomus coccygeum and arterio-venous anastomoses in general, but have failed to substantiate any fundamental similarity between the former and the carotid body. Rather, the present observations indicate that the glomus coccygeum and the carotid body differ sharply from each other in regard to cellular and vascular arrangement, innervation, and cytology.

MATERIAL AND METHODS

Human carotid and coccygeal bodies were obtained at autopsy, from 4 to 6 hours after death. Cat glomeruli caudales and carotid bodies were removed and fixed immediately upon sacrifice of the animal. Fixatives employed were Bouin, Zenker-formol, Regaud's formol-bichromate, and Champy's fluid. The stains used were, (1) Masson's trichrome or a modification thereof, (2) iron hematoxylin, (3) acid fuchsin and methyl green, or (4) Kull's fuchsin, aurantia, toluidine blue. For demonstrating nerve fibers, the pyridine-silver and chloral hydrate modifications of the Cajal technique were used. Many of the tissues were cut and mounted serially. The sections ranged from 3 to 15 μ in thickness.

In the material so prepared, many of the observations already recorded by other workers could be verified. However, as only the more markedly epithelioid³ cell groups of arterio-venous anastomoses bear any resemblance to those of

³ The word "epithelioid" is used throughout this paper in a purely descriptive sense, without prejudice as to the embryological origin or true nature of the cells so described.

the carotid body, study of the glomeruli caudales and glomi coccygei has been directed primarily toward such cell groups. To the beautiful work of Masson (op. cit.), especially, which describes and illustrates the structure of these anastomoses in detail, the present observations have added nothing. While transitional stages between the cells composing the media of the afferent artery and the epithelioid cells composing the media of the arterial segment of the anastomosis were frequently evident, they have not been included in this study. Similarly, glomeruli caudales or portions of the glomus coccygeum with poor epithelioid development were not considered. The material selected for study, therefore, was that which, superficially, bore the most obvious resemblance to the carotid body.

OBSERVATIONS

Cell arrangement and vascularization. As is well known, the epithelioid cells of arterio-venous anastomoses are arranged about the lumen of the vessel, forming the chief component of its media. Sections of a simple anastomosis, therefore, show several layers of cells arranged radially around a single vascular lumen (fig. 1). This is evident both in the glomeruli caudales and in small detached portions of the glomus coccygeum. Sections through the larger portions of the glomus coccygeum often present plate-like groups of cells (fig. 2) with no visible lumen, or with several lumina. Among these are other groups which are sinuous (fig. 3), especially as seen in serial sections. In both of these forms, however, the relation of the cells to the vascular lumina is fundamentally the same as that observed in less complex portions of the glomus. Lobulation within the coccygeal body is thus determined by the grouping of the blood vessels; the smallest lobules, or cell groups, are represented by a section through a single vessel, while the larger are formed by the convolutions of a single vessel, or by the approximation or even fusion of several vessels. The delicate connective tissue characteristic of the

manchon nerveux (Masson)⁴ is frequently evident, especially about isolated vessels (fig. 1).

The vascular lumina present extensive variations, ranging from the contracted condition, in which no lumen is visible, though sometimes marked by the endothelial cells, to greatly dilated channels 50 μ or more in width. Such variations are in line with the known function of arterio-venous anastomoses, and have been previously remarked upon by many investigators. They often profoundly alter the appearance of the tissue.

The cell arrangement of the carotid body varies somewhat with the species studied. In the cat the cells are rather evenly scattered, occurring in small groups, whorls, or cords, or even singly. While these groups are divided by connective tissue and blood vessels, there is no marked lobulation in the carotid body in this animal. The carotid body of the human, however, is less compact, and may be subdivided into lobes and lobules, separated from each other by a variable amount of connective tissue. In cell grouping, therefore, the human carotid body may superficially resemble the coccygeal body. However, the lobules of the carotid body are roughly spherical to fusiform in shape, as followed in serial sections, rather than sinuous. Further, even the smaller lobules contain several anastomosing vessels. In both the cat and the human, the relation between the smaller vascular channels and the epithelioid cells is very close; material in which the vessels are dilated or filled with injection mass supports the conclusions of Schaper (1892) and of de Castro ('26) that every cell within the carotid body is probably in intimate contact with one or more thin-walled blood vessels.

Innervation. Representative sections showing the nerve fibers in portions of the human coccygeal and carotid bodies, as observed in this work, are reproduced in figures 3, 4 and 5. The nerve fibers of the manchon nerveux were best demonstrated about small isolated portions of the glomus coccygeum

⁴ By manchon nerveux, Masson denotes the modified adventitia of the arterial segment of arterio-venous anastomoses. It is composed of interlacing nerve and connective tissue fibers.

(fig. 4). In more compact portions there were numerous nerve fibers around or between the cell groups, but seldom a distinct plexus about an individual group. No nerve fibers could be identified among the cells of the glomus coccygeum (figs. 3, 4) or of the glomeruli caudales.

The innervation of the carotid body and of other similar tissue has been described and figured in recent years by de Castro (op. cit.), Nonidez ('35, '37), Hollinshead ('39, '40 b, '41), and others; the point to be noted here is that from the plexus surrounding a cell group nerve fibers in large numbers penetrate to end upon or between the epithelioid cells (fig. 5). Although the nerve endings are quite delicate, the plexus among the cells is easily demonstrated, and is a characteristic feature of even mediocre silver impregnations. In the cat carotid body, following silver stains, the numerous nerve fibers are so prominent that they may appear to form the chief tissue between adjacent cells and cell groups.

Cytology. The cells of the coccygeal body vary considerably among themselves as seen with ordinary histological techniques. They grade from cells resembling shortened smooth muscle to large, rounded epithelioid elements with clear homogeneous cytoplasm. All of these, however, are in sharp contrast to the ill-defined multiangular carotid body cell of the human, with its large and characteristic vacuoles. The carotid body cells of the cat show much more resemblance to the larger cells of the coccygeal body, as they usually appear rounded in fixed preparations. With most techniques, however, the carotid body cells of this animal present a finely granular cytoplasm, which often also contains small vacuoles, neither of which has been observed in the glomus coccygeum or the glomeruli caudales. All the carotid body cells are of essentially the same appearance, and give no evidence of transitional stages from smooth muscle.

By the use of mitochondrial techniques, many small granules were demonstrated in the carotid body cells. Figure 6 is typical of the human, except that a group with abundant cytoplasm and few vacuoles was selected for illustration. In the

cat, although the granules vary from cell to cell, they are usually very numerous.

In contrast to this, the cells of the glomus coccygeum show only sparse fuchsinophilic granules. Figure 7 represents a cell grouping in which the demonstrable granules are approximately at their maximum. In many of the cells, no granules were visible in 5μ sections, indicating their relative scarcity. They were not definitely identified in the cells of the glomeruli caudales.

DISCUSSION

The present study of coccygeal and carotid bodies, under identical conditions of preparation, has indicated no real similarity between them. In cellular arrangement, vascularization, innervation, and cytology, there are differences which seem to be decisive; these, in part at least, can be clearly related to the present concepts of the functions of arterio-venous anastomoses and of chemoreceptor tissue.

In the glomus coccygeum, the relation between cells and vascular lumen is in accordance with the idea that these cells constitute a large part of the media of the vessel, are apparently derived from the muscular elements, and are presumably contractile. When several channels occur within a single cellular mass, there are still many cells which have no close contact with the endothelium. Masson has interpreted such masses as due to the fusion of several arterio-venous anastomoses, and this seems quite probable. In the present cases, free communications between these vascular channels have not been found; this also is in accordance with Masson's statement that while arterio-venous anastomoses may branch, they do not reunite with each other.

In the carotid body, distinct lobules may or may not occur, as we have seen. In either case, the relation between the cells and the vessels is exceedingly close, a condition of prime importance if the cells are to be affected by changes in composition of the blood. It seems probable that Schumacher ('38) has not appreciated the vascular pattern within chemoreceptor

tissue. For instance, while his figure (Abb. 4, S. 125) of an "abdominal vagal paraganglion" from the mouse presents a vascular arrangement apparently comparable to that sometimes observed in arterio-venous anastomoses, other sections of the same tissue, especially with vascular injections, show numerous anastomosing vessels (Hollinshead, '41, fig. 4).

Of interest in connection with this discussion of vascularity is the statement of Goormaghtigh and Pannier ('39), concerning the blood supply to chemoreceptor tissue. They have described modified vessels, which they interpret as the arterial segment of arterio-venous anastomoses, as a characteristic feature of the carotid body and of the chemoreceptor tissue about the heart. They believe the sole blood supply to chemoreceptor tissue is through such arterio-venous anastomoses, and state that the chemoreceptor cells surround the venous segments of these vessels. According to them, this vascular pattern is a distinguishing feature between motor paraganglia and chemoreceptor tissue (which they interpret as sensory paraganglionic tissue).

The diagrams given by Goormaghtigh and Pannier to illustrate this condition are quite clear, but their figures of observed conditions are not convincing evidence that such anastomoses exist. In the present work, occasional rounded cells have been seen, apparently in the media of some of the smaller vessels in the carotid body, but they are in no way comparable to the pronounced epithelioid development in typical arterio-venous anastomoses in the tail of the cat. Also, the Masson technique has not demonstrated a *manchon nerveux* about any vessel in the carotid body. Further, at least a great many of the smaller, but dilatable, channels among which the carotid body cells are arranged anastomose rather freely with each other; this is certainly not an accepted characteristic of the venous segment of arterio-venous anastomoses, as Goormaghtigh and Pannier recognize in their diagrams. While arterio-venous anastomoses may occur within the carotid body, just as they have been found in other places not previously investigated from this standpoint, it

may be doubted whether such a blood supply is typical and characteristic, as claimed by these authors. Quite recently, Nonidez ('42) has also expressed skepticism on this point.

In regard to the innervation of the structures under comparison, as brought out in the present work, it must be acknowledged that the results probably are not complete. Masson has illustrated much more detail concerning the innervation of arterio-venous anastomoses than has been shown here for the glomus coccygeum. According to this author, many of the nerve fibers are exceedingly difficult to stain. In addition, his drawings of the *manchon nerveux* after silver impregnation were apparently made from sections considerably thicker than those used in the present work, and would also for this reason present a greater complexity of nerve fibers. Masson's description and figures of the innervation of these structures must be accepted as the best to date, as most investigators have been able to bring out much less detail. He has described, but not figured, a "nervous terminal reticulum" among the cells, but one gets the impression, from a reading of his work, that fibers among the cells were usually quite scarce.

On the other hand, all workers who have investigated chemoreceptor tissue with silver stains have remarked upon the richness of the innervation. The intimate association between the cells of this tissue and the nervous system is one of its outstanding characteristics, and would be expected from the standpoint of its known function. The obvious difference in the ease of staining of the fibers in the two tissues is in itself probably an indication of difference in nerve supply; nowhere in the literature, moreover, is there evidence that an innervation similar to that of chemoreceptor tissue exists for the epithelioid cells of arterio-venous anastomoses in general, or of the glomus coccygeum in particular.⁵

⁵ While anatomical studies of arterio-venous anastomoses, as well as clinical studies of tumors arising from them, have indicated that they are abundantly supplied with sensory fibers, these fibers end almost entirely in the adventitia and are believed to respond to changes in size of the vessels. The fibers which must be present among the epithelioid cells, though so rarely demonstrable, are thought to be motor (Masson, *op. cit.*; Nonidez, '42).

In regard to their cytology, the large epithelioid cells of the glomus coccygeum have shown little of interest. Myofibrils have not been observed in them by most workers, and were not seen in the present cases. Indeed, the cytoplasm is usually described merely as "clear"; in the present study, it was stained lightly or not at all, and appeared homogeneous except for the occasional presence of a fine thread of apparent coagulum. The fuchsinophilic granules observed, apparently for the first time, in the cells of the glomus coccygeum are presumably mitochondria. If the none-too-fresh autopsy material used here is regarded as presenting them in approximately their normal numbers, they occur quite sparingly. Failure to observe them in the cells of the glomeruli caudales may be due to the more fuchsinophilic tint usually attained by the cytoplasm of these cells — a tint often associated with the presence of fine myofibrils.

Certain phases of the cytology of the carotid body are at present controversial. Further investigations of this subject are being carried out at present in this laboratory (Hollinshead, '42) and will be reported more fully in a later paper. For purposes of comparison with the coccygeal body, however, it is unnecessary to enter into these more detailed questions. The outstanding features which distinguish these cells are their somewhat granular appearance after bichromate fixation, the presence of vacuoles, especially marked in the human carotid body, and the numerous definite granules demonstrable by mitochondrial techniques. The granular appearance of the cytoplasm with trichrome staining is apparently due to those granules which are brought out more clearly by the mitochondrial techniques; after fixation in a fluid such as Carnoy's, the cell appears empty and coarsely reticular. The vacuoles of the human carotid body, which so frequently markedly distort the cells, have been variously interpreted as true vacuoles and as artefacts, either intra- or extracellular. Many of the very much finer vacuoles in the cat carotid body are obviously intracellular. Regardless of the interpretation

placed upon these vacuoles, they constitute, especially in the human, a very marked difference between the two bodies under discussion. Even if they are regarded as due entirely to post-mortem changes — and Meijling's ('38) description of similar great vacuoles in the freshly fixed carotid body of the horse tends to discredit this — it would be difficult to explain why they should appear in the carotid body and not in the glomus coccygeum, if these two tissues are identical. Finally, the granules here demonstrated in the carotid body by mitochondrial techniques, although in general similar in shape and size to those in the cells of the glomus coccygeum, are far more numerous, frequently almost filling the cytoplasm, and constituting a feature of the carotid body cell which should be considered in any discussion of the intimate physiology of these cells.

While the functional importance of the cytological features described for the carotid and coccygeal bodies may be obscure, the differences between them in regard to vascularization and innervation are more obviously fundamental. The intimate association existing between the individual cells of the carotid body and the vascular stream, on the one hand, and between these same cells and the nervous system, on the other, would seem to be a necessity for efficient functioning of any tissue which is to be influenced by changes in the chemical composition of the blood, and, as a result of such changes, produce reflex actions. The evidence here presented emphasizes the lack of such relationships in the coccygeal body, which resembles rather the anastomoses from the tail of the cat, as studied here, and those from other locations as described in the literature. It therefore seems improbable that the coccygeal body, or other similar tissue, can function efficiently as a chemoreceptor. While hitherto unrecognized chemoreceptor tissue may indeed exist, as pointed out by Guild ('41), it is probably not to be sought in "specialized" arteriovenous anastomoses.

SUMMARY

1. The cells of the glomus coccygeum are arranged in layers about the vascular lumen. Those of the carotid body form thin anastomosing cords or small cell groups separated from each other by vessels, so that each cell is probably in close relation to the blood stream.

2. While cell groups of the glomus coccygeum may be surrounded by a network of nerve fibers, a similar plexus has not been demonstrated among the cells of a group. In silver preparations of the carotid body, however, the nerve plexus is a conspicuous portion of the tissue between adjacent cells.

3. Coccygeal body cells, in their greatest epithelioid development, show little structural detail other than a few fuchsino-phile granules. Cells of the carotid body are distinguished by their numerous granules and the presence of cytoplasmic vacuoles; the latter are an especially prominent feature in the human carotid body.

4. Since the glomus coccygeum and the carotid body apparently do not correspond in vascular arrangement, innervation, or cytology, it is concluded that they are not identical organs. This study gives no support to the view that the glomus coccygeum may be a chemoreceptor.

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PLATE 1

EXPLANATION OF FIGURES

Figures 1 and 2 are unretouched photomicrographs; figures 3, 4, and 5 are photomicrographs upon which the vascular pattern has been emphasized by shading or outline, and the nerve fibers observed under the microscope drawn in with ink. As reproduced $\times 350$. Sections cut at $10\ \mu$.

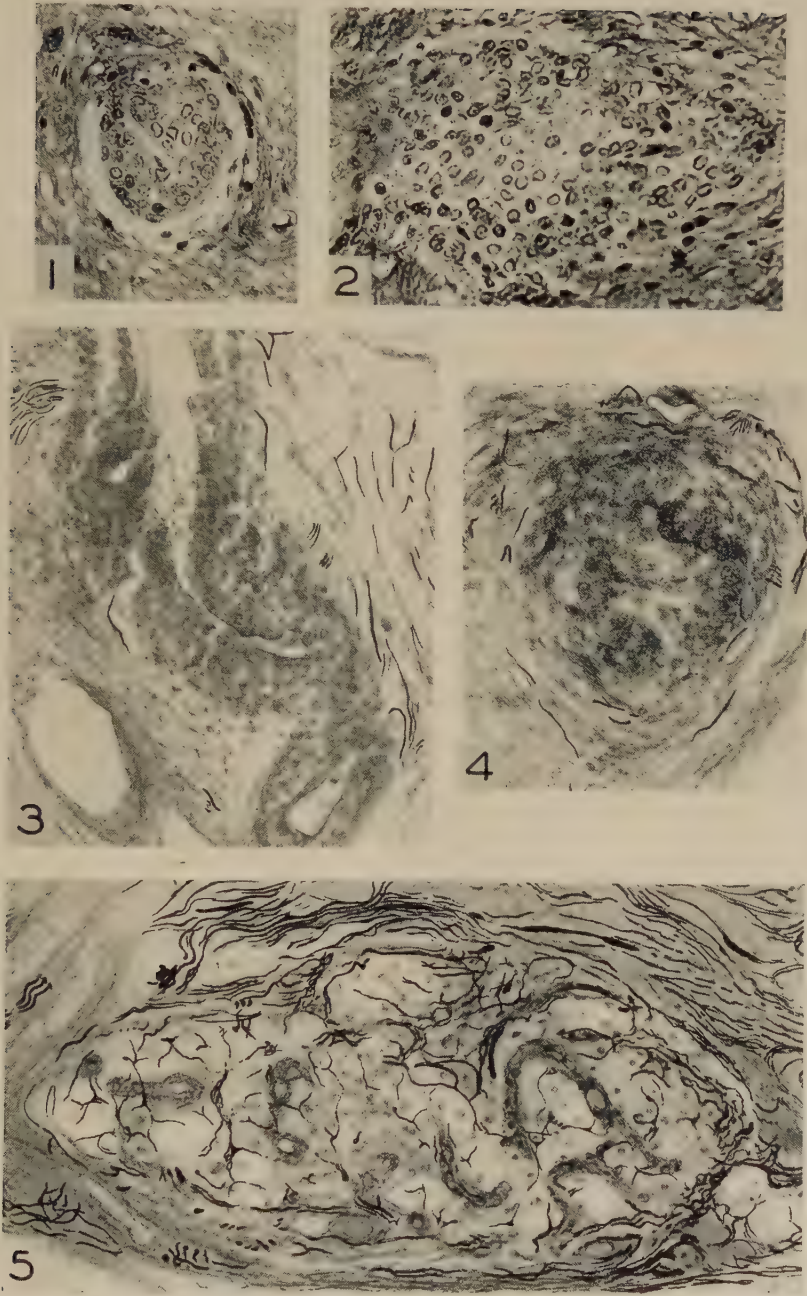
1 Approximate cross section through a simple arterio-venous anastomosis, showing the grouping of the cells about the lumen and the characteristically delicate adventitia surrounding the cell mass. Human coccygeal body, Masson trichrome stain.

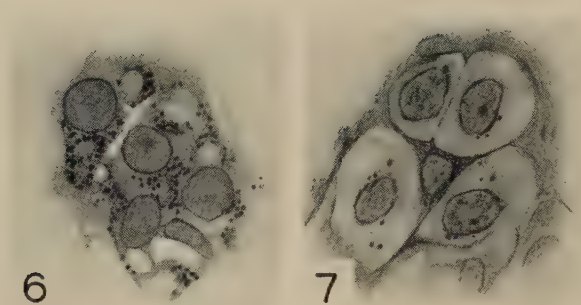
2 Another example of the glomus coccygeum, same stain, showing a larger grouping of cells. In serial sections, several lumina could be traced in this mass.

3 A winding cell-mass with distinct lumen, from a complex grouping of anastomoses in the glomus coccygeum. Nerve fibers are visible in the surrounding connective tissue, but no distinct plexus appears about the vessel. Chloral hydrate and silver.

4 An isolated bit of the glomus coccygeum, with its manchon nerveux of surrounding nerve fibers. Technique as in figure 3.

5 Innervation and vascular pattern in a small lobule of the human carotid body. Pyridine-silver.





Drawings made by Mr. Elon H. Clark, of the Department of Illustration. About $\times 1200$, as reproduced.

6 Vacuoles and fuchsinophilic granules in human carotid body cells. The clearest spaces are either artefacts or capillaries, but several large vacuoles, apparently in the cells, are also present. Acid fuchsin, methyl green.

7 A similar preparation of cells of the coccygeal body. The rather sharp outlines and sparse granulation offer a distinct contrast to the carotid body cells prepared by this technique.

THE OS EPIPYRAMIS OR EPITRIQUETRUM

R. L. DE C. H. SAUNDERS

Department of Anatomy, Dalhousie University, Halifax, Nova Scotia

TWO FIGURES

Accessory or supernumerary carpal bones are not common, but they are of concern to radiologists owing to the fact that they may simulate fracture.

As Köhler ('35) points out the basic work on the subject, that of Pfitzner, unfortunately is somewhat invalidated by the absence of evidence regarding fracture as a possible etiological factor. This, together with the obvious practical need of accurate information on the subject, appears to warrant a report of all such cases when encountered.

DESCRIPTION

The accessory carpal bone here described was found in a dissecting room subject, a male Swedish laborer, aged 70. Fracture as a cause of the condition can be eliminated as it occurred bilaterally, and no pathological features were observed in either the carpal or other joints of both the upper and lower limbs.

Wedged in between the triquetrum, lunate, hamate and capitate, the bone was like a three-sided pyramid, with medial, lateral and distal articular surfaces (fig. 1). The base, which was non-articular, was directed palmar-wards, the apex dorsally. The medial articular facet articulated with the triquetrum, the lateral facet with the lunate, and the distal with the hamate and capitate. These three articular facets were all coated with cartilage, as was the apex of the bone (fig. 2).

The palmar-dorsal length of the right bone was 5 mm., that of the left 6 mm.; the maximum dimensions of the three facets on the right bone were respectively 2, 3, and 4 mm.; on the left bone 3, 3.5, and 4 mm. The smallest facet on each bone was directed towards the triquetrum, the largest distally towards the hamate and capitate.

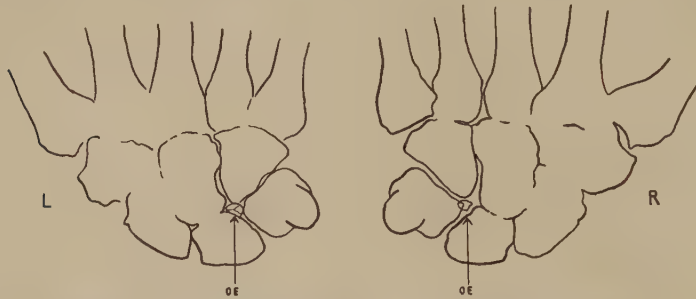


Fig. 1 Tracings from roentgenograms of the dissected hands. The three articular facets are clearly seen in the larger left os epitriquetrum.

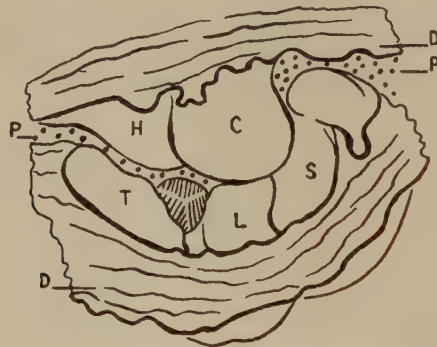


Fig. 2 Dorsal view of the left carpus. The three articular facets (shaded) and apex of the os epitriquetrum are seen. H—hamate. C—capitate. S—scaphoid. L—lunate. T—triquetrum. P—palmar ligaments. D—dorsal ligaments.

A different order of dissection having been followed on the two hands, the right bone was exposed on removal of the palmar carpal ligaments, the left, on removal of the dorsal carpal ligaments. This was fortunate, as it revealed the exact intercarpal relationships of the bones, and showed that in each case the apex was related, but not attached, to a synovial

covered tag projecting into the joint cavity from the deep surface of the dorsal carpal ligaments. The palmar ligaments of both hands found attachment to the rough non-articular base.

Histological section of the smaller right bone, despite poor fixation consequent upon the material coming from the dissecting room, revealed a peripheral fibro-cartilage with a central part or core of cancellous bone.

DISCUSSION

According to Bryce ('15) the os epipyramis or epitriquetrum is depicted by Pfitzner as a dorsally situated ossicle set between the lunate, triquetrum and hamate. Perhaps indicative of its rarity, no reports other than those cited by Ferguson ('33), could be found in the literature. Ferguson only mentions Dwight and Pfitzner as having seen actual specimens; he himself, like Grumbach and Heimerzheim, observed it radiologically. Dwight, although dubious of his specimen, described it as a very rare bone which appeared as a "marking off of the dorsal distal radial angle of the cuneiform [triquetrum]."

The radiograph reproduced by Ferguson ('33) because it seemed a clearer radiographic example than those of the radiologists just mentioned, shows a small ossicle which occupies the same position and presents the same picture as the bone here described and figured. It seems not unlikely, therefore, that the bone visualized by these radiologists may well have been not a dorsally situated element, such as described by Pfitzner and Dwight, but a ventrally placed structure such as the present specimen.

Despite any contrary feeling which these radiologists might have had to such a pronouncement, and the perhaps just assertion that this particular type of ossicle has not been hitherto described, the author does not feel inclined to add a fresh name to an already cumbrous accessory carpal nomenclature. Rather let it be said that this represents a type of

os epipyramis or epitriquetrum, and that therefore a ventral and dorsal type of it may be said to occur.

Before considering etiology, it should be noted that Köhler ('35), on the basis of Grumbach's work, only accepted certain supernumerary carpal bones as true accessory bones. Reserving the term "accessory" for those occurring naturally in the healthy carpus, he excluded all that were doubtful and had either a traumatic or inflammatory origin. As to the epipyramis, he states that Grumbach was undecided whether or not it should be regarded as a true accessory carpal.

Ignoring any possible phylogenetic relationship, it will be granted that their appearance is primarily an ontogenetic problem, and therefore dependent on the pre- and possibly post-natal processes of carpal growth and development.

Let us first consider possible pre-natal factors. If the carpus of a young human embryo (30 mm.) be sectioned coronally (Whillis, '40), it shows the cartilaginous carpal elements separated by mesenchymal joint disks. The subsequent establishment of the intercarpal synovial joint cavities is generally, but erroneously, ascribed to an absorption of the joint disks; but Whillis ('40), correlating joint development with the tissue culture observations of Glücksmann ('39), has pointed out that the intervening mesenchymal joint disks, where subjected to pressure from the rapidly growing carpal elements, undergo a progressive chondrification. So that ultimately (e.g. at the 125-mm. stage) the cartilaginous carpal elements are seen to be united across the joint lines by primitive cartilage.

Solution of continuity is effected about the fifth fetal month by a liquefaction of the primitive uniting cartilage. This temporary but normal cartilaginous union appears to be of importance, and in the present author's opinion it may afford an explanation of non-pathological carpal fusions as well as the occurrence of certain accessory carpals. In short, carpal fusions may be due to a localized failure, while carpal accessories may be credited to a disorderly progress, of liquefaction.

In addition, this suggestion rationalizes these abnormalities in the light of normal growth phenomena, and obviates the necessity of invoking accessory or dual centres of chondrification for each and every new carpal element. It must, however, be admitted in the light of Stockard's work that, when the pre-cartilaginous carpal mesenchyme is about to establish the carpal mosaic, it must be very sensitive and prone to aberration.

Turning then to possible post-natal factors of production, it is strange that the question of metaplasia and heterotopic ossification has apparently not been raised in relation to the genesis of accessory carpals.

Curiosity regarding the synovial covered fibrous tag, related in both hands to the apex of the bone, led to an examination of twenty hands. In all of these inspection of the interval between the triquetrum, lunate, hamate, and capitate, revealed similar tags projecting into the joint cavity from the deep aspect of both the palmar and dorsal carpal ligaments.

Now, as is well known, connective tissue metaplasia is of common occurrence. Fibrous tissue, cartilage, and bone, all being mesodermal derivatives, are closely related, and so one may be changed into the other. Whence it follows that intra-articular structures, such as the tags described above, may in certain instances undergo a metaplasia and give rise to cartilaginous and even osseous accessory articular elements. Von K  lliker, it may be recalled, discovered cartilage cells in the normal villi of synovial membrane.

Whether there is or is not an occupational relationship remains to be decided. It is perhaps not beside the point to mention that Boyd ('34) speaks of the puzzling phenomenon of a loose body occurring in an otherwise normal joint, which is possibly a further, but not inevitable, stage in the development of certain accessory carpal elements.

In this instance the bilateral symmetry of the bone excludes fracture, renders metaplasia possible but improbable, and favors the theory of congenital origin.

SUMMARY

A bilateral os epitriquetrum, composed of cartilage with a core of cancellous bone, was found in the cadaver of a male Swedish laborer, aged 70. Wedged in between the triquetrum, lunate, hamate, and capitate, the accessory ossicles were attached to the palmar carpal ligaments; the left, larger, bone had a maximum antero-posterior length of 6 mm. Discussion of etiology led the author to favor a congenital origin.

I am indebted to Stephen R. Johnston, M.D., for granting radiographic facilities, and to Prof. D. Mainland for helpful criticism.

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HISTOCHEMICAL STUDIES OF THE INTERSTITIAL CELLS OF THE TESTIS

WILLIAM F. POLLOCK¹

Department of Anatomy, Harvard Medical School, Boston, Massachusetts

ONE PLATE (FOUR FIGURES)

This paper presents a histochemical study which attempts to localize in the testis the presumptive testicular hormone, testosterone.

Indirect evidence from a wide variety of physiological experiments has shown that the androgens of the testis are probably secreted by the interstitial cells. From the work of Hanes ('11); Moore and Gallagher ('30); Nelson ('34); Wilhelm and Schwartz ('38); Williams and Cunningham ('40) and numerous others, it appears probable that the testis is capable of secreting androgen so long as the interstitial cells are intact, even though the germinal cells are absent or destroyed.

A lipoid-soluble androgen was separated from fresh bull testis by McGee ('27, '28), testosterone was isolated by David ('35) and synthesized by Ruzicka ('35) and Butenandt ('35), and this substance has come to be regarded as the probable testis hormone. In common with the vast majority of androgens from other natural sources, testosterone is soluble in acetone, alcohol and certain organic solvents, possesses a ketone group, is water-insoluble and forms a semicarbazone (Koch '37, '38, '39).

Presumptive relationships between testosterone and certain other androgens have been developed by Butenandt ('35), Ruzicka ('35 et seq.), Callow and co-workers ('39, '40) Dorfman et al. ('39, '40) and others.

¹ Charles Eliot Ware Memorial Fellow.

The contention of Martins and Rocha ('31) and McCullagh ('32) that there is a water soluble hormone from the testis has not been substantiated and no truly water-soluble testis hormone can be regarded as conclusively demonstrated.

MATERIAL AND METHODS

The phenylhydrazine technique used by Bennett ('40) on the adrenal cortex promised to be of value in localizing testosterone which is chemically similar to the adrenal steroids. Frozen sections of adult cat testes 20 to 200 micra thick were treated with iodine and phenylhydrazine in acetate buffer according to the method of Bennett ('40). Control sections were extracted in alcohol, acetone, petroleum ether or treated with semicarbazide. Sections were similarly prepared from the testes of the adult rat, guinea pig, rabbit and mouse for comparison. The sections were mounted in glycerol-gelatin or glycerol.

The color developed with the phenylhydrazine reagent is seen with least chromatic distortion if daylight is used and this method of illumination is superior to the artificial sources previously used (Bennett, '40). The color of the phenylhydrazine complex is distinct but rather pale when the sections are first prepared; the complex oxidizes to a much darker yellow after the mounted sections have been left overnight.

Because of the possibility that fixation with formaldehyde might yield a false coloration with the phenylhydrazine reagent, if the aldehyde were not completely removed by washing, fresh unfixed material and glands fixed in a mixture of trichloroacetic acid, acetic acid and mercuric chloride were compared with formol fixed sections. There was no difference in any of the sections so prepared and fixation with formol was considered to be satisfactory for these techniques. Fixation in 5% acetic acid was unsatisfactory due to distortion and swelling of the collagen; fixation with picric acid and dichromate fixatives was not feasible due to the color which they impart to the tissues.

Selected sections were also prepared from tissues treated with digitonin (Bennett, '40; Leulier and Revol, '30) and studied with crossed Nicol prisms.

OBSERVATIONS

Sections prepared with the phenylhydrazine technique revealed a yellow color in the interstitial cells of the testis of all the animals examined; the color was not present in the other portions of the testis (fig. 1). The substances responsible for the reaction could be removed by acetone and alcohol, since the color failed to develop in these cells after such extraction (fig. 2). These substances could also be removed by treatment of the sections with semicarbazide.

Treatment with digitonin produced an increase in the number of birefringent crystals in the interstitial cells (fig. 3), but did not diminish the color developed in these cells by the phenylhydrazine reagent after the formation of the digitonides. Treatment with acetone and alcohol removed the normal birefringent crystals from the testis and completely prevented the formation of the digitonides. There is some birefringent material normally present in the tubular portions of the testis, but this birefringence is not increased by treatment with digitonin (fig. 4).

SUMMARY

In the adult cat, as well as in adult rats, guinea pigs, mice and rabbits, substances with solubility properties similar to those of the known active sterones of the testis can be demonstrated in the interstitial cells. They are not found in other portions of the gland. There are birefringent crystals normally present in the interstitial cells, but the number of birefringent crystals can be markedly increased by treatment of the tissues with digitonin, indicating the presence of steroid compounds in the cells. This increase may be due to cholesterol or some similar substance with a trans-3 OH group.

These findings indicate that in the testis of the cat and certain other animals, compounds with the chemical properties

of testosterone are confined exclusively to the interstitial cells and are not detectable elsewhere in the gland. This supplies histochemical evidence supporting the physiological evidence that the interstitial cells are the source of the testicular androgen.

The author wishes to express his sincere gratitude to Dr. H. Stanley Bennett for his understanding guidance and criticism.

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PLATE 1

EXPLANATION OF FIGURES

1 Frozen section of formol-fixed cat testis stained with phenylhydrazine and mounted in glycerol-gelatin. The photograph was taken by reflected light through a blue filter to accent the yellow of the phenylhydrazine complex, which shows here as the dark material in the interstitial cells. $\times 90$.

2 Frozen section from the same gland, treated exactly the same as that shown in figure 1, except that the section was extracted in acetone overnight before being treated with phenylhydrazine. The section was photographed by reflected light through the same filter used in taking the picture shown in figure 1. Note that the dark phenylhydrazine complex is absent from the interstitial cells, showing that the substances responsible for the reaction are soluble in acetone. This section is similar in appearance to those extracted with alcohol or treated with semicarbazide before treatment with phenylhydrazine. The prevention of the formation of any phenylhydrazine by semicarbazide indicates that carbonyl groups are responsible for the yellow color seen in figure 1. $\times 90$.

3 Frozen section from the same gland, treated with aqueous digitonin before sectioning. The photograph was taken through crossed Nicol prisms. The birefringent crystals probably consist principally of cholesterol esters mixed with digitonides of substances with a phenanthrene nucleus and a trans-3-hydroxy group. Dehydroisoandrosterone fulfills the latter requirement. $\times 90$.

4 Frozen section from the same gland, mounted in glycerol-gelatin without digitonin treatment. This shows the birefringent crystals normally present in the testis; probably most of them are cholesterol esters. $\times 90$.



PARTICULATE GLYCOGEN: A SUBMICROSCOPIC
COMPONENT OF THE GUINEA PIG LIVER CELL;
ITS SIGNIFICANCE IN GLYCOGEN STORAGE
AND THE REGULATION OF
BLOOD SUGAR ¹

ARNOLD LAZAROW

Department of Anatomy, University of Chicago, Illinois

INTRODUCTION

The advent of the ultracentrifuge has given great impetus to the study of ultramicroscopic structures found in animal cells. Doctor Claude ('40) at the Rockefeller Institute and Professor Bensley and myself at the Hull Laboratories recently have been studying these ultramicroscopic structures.

We have found ² that the cytoplasm of the guinea pig liver contains two distinct submicroscopic particulates in addition to the mitochondria previously separated by Bensley and Hoerr ('34). One of these, particulate glycogen, is the subject of this paper. The second is a lipo-protein complex. This ultramicroscopic lipo-protein complex differs in chemical composition from the microscopically visible mitochondria previously analyzed by Bensley ('37). (The latter also contains proteins and fats.) Furthermore, the lipo-protein ultramicroscopic particles as well as the visible mitochondria contain some of the respiratory enzymes; Lazarow and Barrón ('41, also unpublished data). The enzyme phosphatase has been found by Kabat ('41) to be associated with the lipo-protein ultramicroscopic particle. Claude found that the chick virus

¹ This work has been aided by a grant from the Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

² Data soon to be published.

tumor principle was associated with this normal ultramicroscopic components of cells. These facts suggest that the particulate component of protoplasm play an extremely important role in the physiological activity and organization of the cell.

HISTORICAL CONSIDERATION OF THE DISTRIBUTION OF GLYCOGEN IN THE CELL

Subsequent to his discovery of glycogen in 1857, Claude Bernard (1859) studied, by means of the iodine reaction, the distribution of glycogen in various tissues including liver, placenta, and various embryonic tissues. He described, for example, plaques of cells in the amniotic membrane of the calf containing amorphous and round granules of a brown staining material and pointed out their similarity to those of liver cells.

At the same time that Claude Bernard announced his separation of glycogen, Schiff (1857) erroneously announced that he could identify glycogen in the unstained liver as granules. These granules, which were clearly differentiated from fat droplets, were said to be abundant in glycogen-rich livers and scarce in livers containing little glycogen. He further claimed that they completely disappeared by digestion with amylase. However, Bock and Hoffmann (1872), showed that the granules described by Schiff were present in livers containing no glycogen whatever and that after staining with a solution of iodine in potassium iodide a diffuse brown staining network could be seen between the granules of Schiff, which themselves remained colorless. Furthermore, the iodine reaction disappeared after salivary digestion. Even before Bock and Hoffmann, Rouget (1859) said that in cartilage and muscle, the glycogen existed as a "plasma" and not as a granular substance.

Heidenhain (1883), referring to the work done in his laboratory in 1879 by Richard Kayser, states that in alcohol fixed sections, the glycogen exists in the form of granules and flakes regularly located upon one side of the cell. However, he does

not state whether he considers this distribution as an artifact of the alcohol fixation. Külz (1881) as well as many authors following him, Afanassjew (1883), Barfurth (1885), Best ('03); and Fischera ('04), pointed out that this granularity, localized at one side of the cell, was an artifact of the alcohol fixation.

To Ehrlich (1883), however, belongs the credit of conclusively proving that glycogen is uniformly distributed throughout the liver cell in a diffuse state. He reasoned that, if the glycogen granules were preformed, they should be demonstrable in the dried preparation. However, upon staining a dried smear of liver with iodine he observed a homogeneous brown staining of the cytoplasm; whereas the nucleus contained no brown color. This diffuse distribution of the glycogen was confirmed for cells in tissue culture of *Fundulus* embryo by Lewis ('21), and for liver by Nordman ('29). Gersh ('38), by means of the freezing-drying technique, obtained the same results that Ehrlich did.

The separation of glycogen in the form of submicroscopic particulates, in no way contradicts any of the above observations. A uniform distribution of the submicroscopic component throughout the cytoplasm of the liver cell would give a diffuse appearance when studied by the freezing-drying method. However, if glycogen possesses the ability to form submicroscopic³ aggregates, it may also be able to form microscopically visible structures. Marchand (1885), conceded the diffuse distribution of glycogen in liver, but observed vacuoles of glycogen in dried preparations of leucocytes. Similarly, Gierke ('05), observed glistening balls in fresh cartilage cells which corresponded to the location of the glycogen after fixation. He also stressed the great regularity of orientation of glycogen granules in the kidney tubules of the cat. Similar orientation in the uterine epithelium is described by Bartelmez and Bensley ('32), but these authors

³ This submicroscopic particle, as will be shown later, consists of an aggregate of smaller glycogen units. Upon dispersion, this particle assumes the properties of glycogen, prepared by the method of Pflüger.

state they are uncertain as to whether the granules are pre-formed or are the result of plasmolysis. However, Lewis and Nordman observed that in the process of staining by iodine vapor the cells in the tissue culture, which previously showed a diffuse browning, would, at times, suddenly develop blebs or granules of brown staining material. Nordman, at times, observed these droplets in the plasma outside the cells. These transformations were only observed during the stage of cell death. This observation might be taken, at first glance, to suggest that all vacuoles are secondary to cell death; however, since iodine-potassium iodide solution is a strong protein coagulator,⁴ these changes may well be the result of iodine coagulation. They do not necessarily account for the vacuolar appearance seen in certain fresh or dried cells.

It is of interest to note that Cori, Cori and Schmidt ('39), found that the glycogen, synthesized by an enzyme prepared from muscle, increased the turbidity of the solution and resulted in a granular precipitate visible under the microscope.⁵

It is clear, therefore, that glycogen exists as a uniformly distributed substance in the liver cell. We have separated it in the form of a submicroscopic particle. Whether it also exists in a microscopically visible form in liver and other cells awaits further study.

SEPARATION OF THE PARTICULATE GLYCOGEN

Adult guinea pigs, allowed excess food for the previous 24-48 hours were killed by a blow on the head. The thoracic cavities were opened, and by way of the descending aorta, the animals were perfused with cold saline. After perfusion, the

⁴ If one adds a solution of I-KI to a liver extract freed of all the microscopic and submicroscopic granules the protein is promptly coagulated and forms a precipitate.

⁵ No granules, however, resulted from glycogen synthesis when the enzyme was prepared from other sources (liver and brain). Since myosin has been shown by Przylecki and Majmin ('34) to form precipitated complexes with glycogen, whereas albumens and globulins, at physiological pH's do not, Przylecki, Andrzejewski and Mystkowski ('35), it is not unlikely that the granular precipitate of glycogen, synthesized by the muscle enzyme is due to contained myosin in the enzyme preparation.

livers were removed and fragmented by forcing them through surgical gauze and finally through bolting silk. This procedure fragments a large percentage of the cytoplasmic membranes of the cells, thus dispersing most of the cytoplasmic constituents. The nuclear membranes, however, are not easily disrupted and most of the nuclei remain intact. A few intact cells also remain. This liver emulsion was suspended in three times its volume of 0.85% sodium chloride and fractionated by differential centrifugation in a cold room at 2–3°C.

The suspension was clarified for two 10-minute intervals at 3000 R.P.M. in an 8-inch angle centrifuge and then for 15 minutes at 6000 R.P.M. The above⁶ precipitates were discarded. The supernatant was then transferred to clean 10 cc. lusteroid test tubes, and centrifuged at 12,000 R.P.M.⁷ for 30 minutes. The precipitate consisted of two parts: (a) a densely packed cake which remains inclined in the tube corresponding to its position on centrifugation—this is the particulate glycogen; (b) a loosely packed, red colored, precipitate which is easily separated from (a) by inverting the test tube. This latter precipitate is a mixture of particulate glycogen and the lipo-protein submicroscopic cell component. The surface of the tightly packed cake is washed twice with saline and then resuspended in saline, allowing 30 minutes for dispersion. It is then recentrifuged at 12,000 R.P.M. for 30 minutes and all but the tightly packed cake is discarded. The above procedure is repeated four times with saline, or with distilled water if desired. This cake upon being dried in a vacuum desiccator yields a pure white powder. Chemical analysis shows this powder to consist mainly of glycogen.

METHODS OF CHEMICAL ANALYSIS

The jelly-like cake was dried in a vacuum desiccator over P_2O_5 for 24–48 hours and then extracted in a Soxhlet extractor for a period of 6 days (48 hours with each of the following:

⁶ These precipitates contained nuclei, mitochondria, and some submicroscopic components.

⁷ This corresponds to a gravitational force of about 12,000 G.

redistilled alcohol, ether and chloroform). The above three extracts were combined, evaporated to dryness and re-extracted with chloroform. The fats go into solution, leaving a considerable residue behind which is water soluble. This residue is composed largely of salts but contains other organic extractives. This method of extraction is fairly efficient as only 0.3% ash remains in the original alcohol-ether-chloroform (A-E-C) insoluble residue.

Water. In some cases the approximate quantity of water in the original jelly was estimated by comparing the chloride concentration of the dried jelly with that of the saline used for extraction. The chlorides were determined gravimetrically. This, of course, assumes that the salts are uniformly distributed throughout all the water of the particle. In other cases the water was determined directly by weighing the jelly before and after drying. Obviously, these methods of water analysis are only approximate. The water percentage does not represent the true water content of the particle; for if we assume that the glycogen particles are spheres and that they are packed as closely as their configuration permits, some of this water lies outside the particle. A calculation shows that 26% of the water would be extra-particulate if the above assumptions were correct.

Fats. The chloroform soluble fraction was dried in a vacuum desiccator for 24 hours and the fats weighed on a micro-balance.

The A-E-C insoluble residue was analyzed for carbon, hydrogen, ash, nitrogen, phosphorus, glucose, and protein. Micro carbon, hydrogen, and ash analyses were performed by Doctor Ma of the Department of Chemistry by the micro Pregl method ('37). The nitrogen was determined by micro Kjeldahl digestion followed by direct Nesslerization as given by Koch ('37). The phosphorus was determined by a modification of the Fiske and Subbarow method ('25), using higher acidity and heat to develop the blue color.

Glycogen. The glycogen was determined as glucose following a 30-minute period of hydrolysis with 3 N. H_2SO_4 . The

glucose was determined by the micro method of Miller-Van Slyke ('36). The glucose value multiplied by the factor 162/180 gives the glycogen content.

Protein. An attempt to determine how much of the total nitrogen represents protein was not entirely successful. Proteins are precipitated by trichloroacetic acid (T. A. A.). If the nitrogen is determined in the T. A. A. filtrate and precipitate, the ratio of protein nitrogen to total nitrogen can be calculated. However, when the percent of protein is very low and is present in a complex, as is the case in our material, not all of the protein is coagulated by cold T. A. A., and part remains in solution. But, if the T. A. A. is heated for 30 minutes in a water bath the protein is aggregated into large clumps. Heating with 4% T. A. A. for 30 minutes hydrolyzes some of the protein.⁸ In spite of this fact, more protein was recovered from the glycogen sample after heating than was recovered from precipitation in the cold. Although the method for protein estimation is inadequate, we can show that the nitrogen in the sample, in large part at least, represents protein nitrogen.

PROPERTIES OF PARTICULATE GLYCOGEN

General physical properties. The moist centrifuged cake has a jelly-like consistency. It is transparent and has a white or faint yellow color. When viewed by reflected light it is moderately opaque. If the cake is allowed to stand for a short time in 0.85% sodium chloride, or water, it disperses and produces a markedly opalescent solution. This solution is fairly stable for many days, but can be precipitated by 50% alcohol. It redisperses upon adding water.

Microscopic properties. A suspension of particulate glycogen, when studied under the microscope with an oil immersion lens shows no structure whatever. When studied with an ultramicroscope, small particles, undergoing rapid Brownian movement, are distinctly visible.

Centrifugability. The particulate glycogen may be completely removed from solution by centrifuging in an angle

⁸ Using similar amounts of crystalline egg albumen, about 25% of the protein is hydrolyzed and appears in the T. A. A. filtrate.

centrifuge for 30 minutes at 12,000 R.P.M. (This corresponds to a centrifugal force of 12,000 G.) When centrifuging commercial glycogen (Pfanstiehl) for the same length of time 99.7% of the glycogen remains in solution. However, the particulate glycogen begins to sediment at speeds as low as 3000 R.P.M. After a 40-minute centrifugation at this speed the glycogen is seen to have settled in a fairly sharp boundary. The upper 10 mm. of the tube showed no opalescence and contained no glycogen, whereas the rest of the tube appeared markedly opalescent. Using the Svedberg ('40), definition of sedimentation constant as the rate of sedimentation expressed as centimeters per second when the sedimenting force is one dyne, we obtain an approximate value of $4,200 \times 10^{-13}$ for particulate glycogen. A comparison with table 1 shows

TABLE 1
Particle weight and sedimentation constants of various proteins.

	$K_s \times 10^{-13}$	M.W. (MILLION)
Serum albumen (horse) ¹	4.5	0.067
Edestin ¹	12.8	0.303
Erythrocrucorin (Planoribis blood) ¹	33.7	1.6
Hemocyanin (Helix Blood) ¹	98.9	6.6
Tobacco Mosaic Virus ²	191-244	15-20
Particulate glycogen	4,200 (approximate)	?

¹ Taken from Schmidt ('38) pp. 393-394.

² Taken from Eriksson-Quensel and Svedberg ('36).

that the particle weight of particulate glycogen is much greater than even that of Tobacco Mosaic virus. It is also of interest to compare the particle weight of the former with glycogen prepared in the usual way. Oakley and Young ('36), estimated by means of an osmotic pressure method, that glycogen prepared by KOH extraction had an average molecular weight of two million. Mystkowski ('37) found that similar preparations of glycogen began to sediment at 17,000 R.P.M. Since the particulate glycogen begins to sediment at 3000 R.P.M., whereas, the same glycogen, after dispersion with KOH, cannot be removed by the maximum speed of our

centrifuge (12,000 R.P.M.), it is clear that the particle represents an aggregate of many smaller glycogen units.

Dispersion of the particulate glycogen. The particulate glycogen can be dispersed by any of the agents commonly used for the separation of glycogen from the cell. Four per cent Trichloroacetic acid or 25% NaOH produced a marked decrease in the turbidity of the solution as well as a loss of the centrifugability. A control test was performed by adding sodium chloride to the particulate glycogen suspension to produce a corresponding increase in density but this showed no loss of centrifugability. Similarly, heating for 2 hours on a steam bath produced a marked decrease in the opalescence of the solution and 94% of the glycogen particles were dispersed. The remaining 6% could still be recovered by the centrifuge. Incubation for 2 hours at 37°C. showed no dispersion of the particle.

CHEMICAL PROPERTIES

The glycogen gives a positive Molish test, and a brown to red color with iodine. The latter disappears upon heating and reappears upon cooling. It reduces Fehlings solution only after hydrolysis.

TABLE 2
Analysis of the moist product.

	SAMPLE ¹ C	SAMPLE ² D
	%	%
Water (approximate value)	73	73
Solids	27	27

¹ H₂O estimated from salts.

² H₂O estimated by drying.

TABLE 3
Analysis of the dried material.

	SAMPLE A	SAMPLE C
	%	%
Salts and other water soluble extractives	1.50 ³	2.57
Fat (chloroform soluble)	0.16	0.165
Residue (A-E-C insoluble)	98.34	97.26

³ The particulate glycogen in this case was washed once in distilled H₂O before drying.

It is to be noted that sample A (see table 4) which was dried in a vacuum desiccator, after extraction of the fats and salts, still showed evidence of residual moisture content, for the percentage of hydrogen was high. However, upon heating sample C at 100°C. for 24 hours and then drying in a vacuum desiccator a considerable portion of the water was lost, as evidenced

TABLE 4
Analysis of the alcohol-ether-chloroform insoluble residue.

	SAMPLE A (DRIED OVER P_2O_5 IN VACUUM DESICCATOR)	SAMPLE C (DRIED AT 100°C. 2 HRS. AND THEN PLACED IN VACUUM DESICCATOR)	$C_6H_{10}O_5$	COMMERCIAL GLYCOGEN DRIED IN VACUUM DESICCATOR
	%	%	%	%
Carbon	41.67	41.50	44.56	...
Hydrogen	7.78	6.46	6.22	...
Ash		0.37		...
Glycogen	92.2	93.5		93.2
Phosphorus	0.033	0.019		...
Nitrogen	0.23	0.17		...
Protein nitrogen (T.A.A. ppt.)		0.102		...
Protein (maximum)		1.07		...
Protein (minimum) ¹		0.86		...

¹ Determined by correcting T.A.A. ppt. nitrogen for hydrolysis and multiplying by 100/15.9.

by much lower hydrogen values. Slater ('24) actually succeeded in preparing a mono-hydrate of glycogen ($C_6H_{10}O_5 \cdot H_2O$)_n which when dried in a vacuum desiccator over $CaCl_2$ gave up only 50% of its moisture content. The resulting hemi-hydrate loses about 30% more of the original water content upon heating at 110°C. for 1-2 hours. It takes 9 days, however, to lose most of the remaining water.

From some of our experimental data it seems that this peculiar hydration property may be related, in part, to the absence of salts. The original particulate glycogen was separated from salt solution and was dried in a vacuum desiccator

over P_2O_5 . However, after extraction with alcohol, ether, and chloroform, it showed a 6.95% increase in weight when dried under identical conditions. Upon heating at $100^\circ C$. for 24 hours part of this apparent gain in weight was lost. The extraction removes most of the salts. The evidence previously presented, with respect to the hydrogen content, suggests that this increase represents water. By electrophoresis studies glycogen has been shown to have a negative charge (Samec and Isajevic, '23; and McBride, '29). In the presence of salts, the ions may serve to maintain electrostatic equilibrium. In the absence of salts, however, utilization of the water dipoles may be required to accomplish this purpose and hence the difficulty in removing the residual water.

THE PREEXISTENCE OF THE SUBMICROSCOPIC PARTICULATES IN THE CELL

Do these isolated glycogen particulates exist as such in the cell or are they the artificial result of the procedure used in separating them? If these particles were artificially formed by the conditions of the separation, addition of dispersed glycogen to the supernatant (out of which the glycogen particulates were originally obtained) should result in aggregation and development of more particles. Both particulate glycogen, dispersed by heat, and commercial glycogen were added to the supernatant and incubated for 3 hours at $37^\circ C$. and $0^\circ C$. There was no evidence of particulate formation—that is no change in turbidity of the solution and no appearance of particulates upon centrifugation.

The conditions of the separation involve no drastic alteration of pH or protein precipitating agents. The only change is from the ionic environment of the cell to that of 0.85% NaCl. An attempt was made to separate the particulate glycogen in salt solutions approaching intracellular fluid.⁹ The following solutions were used: (a) KCl-MgCl₂-phosphate

⁹ Hastings and his associates have suggested that intracellular fluid is rich in potassium, magnesium, and phosphate and poor in chloride. See Manery and Hastings ('39).

mixture, and (b) potassium phosphate buffer, pH 7.2. Particulate glycogen was separated in both cases when these salt solutions were used for fragmentation of the liver cells.

The above findings, taken with the fact that particulate glycogen is dispersed by all the agents commonly used to separate glycogen from the cell (heat, trichloroacetic acid, and KOH), strongly suggests that it is present as such in the cytoplasm of the cell. The demonstration of the centrifugability of glycogen within the liver cell may furnish more direct proof of the preexistence of the glycogen particles. This experiment will be undertaken.

DISCUSSION

Water content. The separation of the particulate glycogen containing water is of interest because of the various reports in the literature on this question. Fenn ('39) reviewed the literature on this subject. He presents good evidence that glycogen is stored in the cell along with water, potassium, and acid soluble phosphate. This is concluded from the fact that as the glycogen content of the whole liver increases, the water content, potassium, and acid soluble phosphate show a parallel increase, whereas the protein content remains unchanged. The actual percentages, however, of water, potassium, and acid soluble phosphate remain relatively unchanged, whereas the percentage of protein decreases. Glycogen storage is, of course, associated with an increase in weight of the liver. From this data Fenn concluded that for each gram of glycogen, 2-3 gm. of water were also stored in the liver.

Protein content. The isolation of a glycogen particulate complex containing a small amount of protein is of special interest. Combinations of protein and glycogen have been known for some time. Przylecki and Majmin ('34), and Mystkowski, Stiller and Zysman ('35), showed combinations of glycogen with myosin and myoglobin. Mystkowski ('37), by means of an ultracentrifugal study, has shown a combination of serum globulin and glycogen; the addition of glycogen produces a 20% apparent increase in the molecular weight of

the globulin. He failed to show any combination with albumen. Willstätter and Rohdewald ('34), who reviewed the early literature on this subject, found, by isoelectric precipitation of a liver extract, that they could obtain a protein fraction containing glycogen. Aubel, Reich, and Lang ('38), were able to isolate a similar complex by ammonium sulphate precipitation of the proteins. This contained 20% glycogen. Wajzer ('39), in studying the amount of glycogen protein complex in the liver of frogs, was able to recover from 46–94% of the total liver glycogen by trichloroacetic acid precipitation of liver extracts. None of these authors, however, report a glycogen percentage in any way approaching the value of our complex.

If all the nitrogen in our preparation represents protein, the total protein amounts to only 1–1.4%. It would appear, at first glance, that this might be a contaminant — especially since this particulate glycogen must be separated from a second submicroscopic lipo-protein complex. But the percentage of fat in the glycogen sample amounts to only 0.16%. The lipo-protein complex contains about 40–50% fat and 50–60% protein. Therefore, if all the fat present were due to the lipo-protein complex contamination, the protein contamination would be approximately 0.16%. This figure is but a small fraction of the actual protein found.

This particulate glycogen is dispersed by heat, trichloroacetic acid, and alkali. None of these agents is said to affect glycogen, whereas they all radically modify proteins. This suggests, therefore, that the protein content, although small, plays an important role in the particulate structure. It is not unlikely that the protein complexes isolated by the above authors are mixtures containing the particulate glycogen and protein.

Significance of particulate glycogen in the problem of insoluble glycogen. The protein content assumes further significance when considering the problem of the solubility of glycogen in various tissues. Ehrlich, as early as 1883, was aware that the glycogen of different tissues dissolves at different

rates. For example, he states that the glycogen of liver sections readily dissolves in water; the glycogen in cartilage is dissolved with much greater difficulty; that in stratified epithelium is not dissolved at all. Not only did he point out that a second substance, which he called "Träger Substance" might be responsible for this variation in solubility, but he suggested that this might be a protein. Külz (1886) made a similar suggestion. The difficulty of removing all the glycogen from muscle by aqueous extraction is a well known fact.

Fränkel (1892), stated that freshly ground liver, or liver which was coagulated with alcohol, dried and powdered, gave no glycogen when extracted with cold water. If the same preparations were extracted with hot water, acids, or salts of heavy metals, a cloudy solution containing glycogen was obtained. He considered this data as proof of a protein complex and of the liberation of glycogen by protein coagulating agents. Saake (1892), however, pointed out that these results could also be interpreted on the basis of alterations of permeability of the cell wall brought about by the above agents.

The separation of particulate glycogen throws more light on the problem of insoluble glycogen. Whether or not these particles can get out of the cell would depend upon the permeability of the cell wall. By our method of fragmenting liver, most of the cytoplasmic membranes are broken. Hence, the particulates are liberated, even in ice water. However, if the liver is simply ground large numbers of cells would remain intact and cold water could not be expected to remove the glycogen. The application of heat and acid not only alters the permeability, but also dissociates the particle. This liberates the glycogen from the cell. Other cells such as cartilage and stratified epithelium may hardly be broken at all by grinding, and consequently give little glycogen by simple aqueous extraction.

The case of muscle is especially interesting. It is relatively difficult to break up muscle fibers mechanically. If the particulates are present in muscle as well as in liver they might not easily be liberated. Furthermore, since the myosin

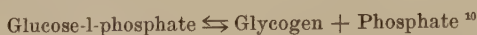
extracted from the cell is precipitated at pH 6.9–7, it is easy to understand why, at the pH of the cell, the cytoplasm of broken muscle cells shows little tendency to disperse. Meyerhoff ('30) pointed out that in frog muscle only one-sixth of the muscle glycogen could be obtained in the pressed muscle juice. Furthermore, Przylecki and Majmin ('34), and Mystkowski, Stiller and Zysman ('35), pointed out that myosin forms definite complexes with glycogen and that preparations of myosin and myoglobin-X contain measurable quantities of glycogen. These authors state that in the muscle cell the myosin and myoglobin are intimately associated with the glycogen. It remains to be investigated whether or not submicroscopic aggregates of glycogen exist in muscle and what their relationship is to myosin and the structure of muscle.

Phosphorus content. Part of the phosphorus content of our preparation may also be explained on the basis of the protein content. Other proteins which were separated from guinea pig liver contain phosphorus. Sahyun and Alsberg ('30) reported glycogen samples giving no phosphorus directly, but giving about 0.03% after hydrolysis. McDowell ('22) was unable to free glycogen from phosphorus by prolonged electro-dialysis. Hassid and Chaikoff ('38), using purified glycogen preparations, obtained by trichloroacetic acid precipitation, report a much lower percentage of phosphorus (0.001–0.01%). From our previously mentioned experience in attempting to determine the protein nitrogen by trichloroacetic acid precipitation it is clear that all the protein cannot be removed from the glycogen by cold trichloroacetic acid.

Significance of particulate glycogen in glycogen storage and the regulation of blood sugar. It is clear that glycogen exists in the liver in the form of submicroscopic particles. These particles, which are aggregates of smaller glycogen units, are very stable at 37°C. but they can be dispersed by drastic treatment, such as prolonged heating at 100°C., strong alkali or acid. Although they contain a very small amount of protein, this protein may be of great importance in the maintenance of glycogen in the particulate state inasmuch as all the agents

which disperse the particulate glycogen markedly alter protein. None of these is thought to alter the properties of glycogen.

It is clear that, if this protein, or some other agent, combines with the dispersed glycogen as the latter is synthesized in the liver cell, the glycogen will be removed from solution by the formation of particulates. By the law of Mass Action the enzymatic reaction



would be shifted in favor of glycogen synthesis, therefore, facilitating glycogen storage in the liver. The concentration of glucose in the liver cell would be diminished. This would favor the removal of glucose from the blood stream and a consequent lowering of blood sugar.

If this coacervating agent were insulin, or, if insulin catalyses this reaction, it could explain one mechanism of insulin action, for we know that insulin effectively lowers the blood sugar and facilitates glycogen storage. Whether it is insulin or not, a simple defect in the mechanism of particulate glycogen formation within the liver cell would result in a decrease in glycogen storage and an elevation of the blood sugar. If the renal threshold were exceeded a glycosuria would develop and a "diabetic like" state would result. All of this could be the result of a primary defect within the liver cell — i.e., a liver diabetes. Thus one type of diabetes could be the result of a defect in the regulatory mechanism within the liver cell and still could be associated with a "normal" utilization of sugar, though at a higher blood level, and "normal" rate of conversion of fats and proteins into sugar. If insulin were the agent which controls particulate glycogen formation, then pancreatic diabetes could be the result of a primary failure in this regulatory mechanism. This is in direct contrast to the non-utilization and overproduction theories for the cause of pancreatic diabetes.

Investigations are now in progress to determine the nature of the protein contained in particulate glycogen, the factors

¹⁰ The enzyme glycogen phosphorylase, which catalyzes this reaction has been isolated by Cori, Cori and Schmidt ('39).

involved in particulate glycogen formation, and the state of glycogen in various types of experimental animals.

CONCLUSIONS

1. A submicroscopic complex of glycogen has been isolated from the liver of guinea pigs by means of a high speed centrifuge.

2. This complex contains a high percentage of water and lends support to the concept of storage of water with glycogen in the cell.

3. It contains 92-93.5% of glycogen on a dry weight basis.

4. It contains a small percentage of protein (approximately 1%) but this may play a very important role in the maintenance of the complex.

5. It contains a small amount of nitrogen and phosphorus. Most, if not all, of the nitrogen as well as part of the phosphorus is due to the protein content.

6. The particles are stable at 37°C. but may be dissociated into smaller units by heat, trichloroacetic acid and KOH (common agents used in glycogen preparation).

7. The significance of the particulate glycogen in the process of glycogen storage in the liver and the regulation of blood sugar has been discussed.

I should like to express my deep appreciation to Prof. R. R. Bensley for his suggestions, criticisms, and constant encouragement throughout this work.

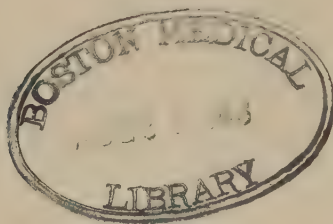
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THE MOUSE ADRENAL

I. DEVELOPMENT, DEGENERATION AND REGENERATION OF THE X-ZONE ¹

M. K. McPHAIL AND H. C. READ

Department of Pharmacology, Dalhousie University, Halifax, N. S.

THREE PLATES (TWELVE FIGURES)

INTRODUCTION

The presence of a distinctive zone, the X-zone of Howard or the androgenic zone of Grollman (Grollman, '36), at the cortico-medullary boundary of the adrenal gland of the pre-puberal mouse is well known; its origin and mode of differentiation from the rest of the gland, however, are not satisfactorily understood. Howard ('27 and '38) who has made a careful study of this area was first of the opinion that it is a differentiation of the adult cortex, developing only after birth and being clearly defined at 3 weeks of age. Waring ('35) from an examination of the adrenals of embryos concluded that it is a transitory development of the embryonic cortical anlage. He found that the cortical cells began to separate into a glomerulosa and fasciculata by the fifteenth day of intra-uterine life and that as this differentiation proceeded certain of the cortical cells gradually became isolated to a region immediately surrounding the developing medulla. At birth this area is only 1 or 2 cells deep but can be demarcated from the cells of the permanent cortex by their deeply eosinophilic properties. It is this narrow border of cells that in

¹ We are indebted to the Banting Research Foundation for a personal grant to one of us (H.C.R.).

post-natal life proliferates to become the X-zone. Howard ('39) accepts Waring's view in part but adds "in my experience the differentiation of the X-zone in the mouse adrenal in the first ten days of extra-uterine life is normally so indefinite that on the present evidence I am unable to concur entirely with Waring's conception."

We have reinvestigated the changes that occur in the adrenal during its early development and this with certain studies on the degeneration and regeneration of the X-zone are recorded below.

MATERIALS AND METHODS

The mice used in the investigation were obtained from a heterozygous inbred colony maintained in the department for the past 2 years. Although predominantly white, brown, black and piebald animals occurred and all were used. The strain apparently is high in X-zone tissue (Howard, '38) as we have never failed to find good development of the zone in several hundred prepuberal mice examined.

Unless otherwise stated, the animals were killed by ether or chloroform, the adrenals fixed in Bouin's fluid, embedded in paraffin and sectioned at 6 μ . Ehrlich's haematoxylin and eosin were used as routine stains and special methods used only for certain parts of the work; e.g. formol-saline, Ciaccio, osmic acid and Susa were used as fixatives, and Mallory's, Heidenhain's iron haematoxylin and Masson's haematoxylin-ponceau-fuchsin-light green used as stains.

The pregnancies were dated as from the finding of a vaginal plug; the animals being examined daily each morning.

The testosterone propionate² and colchicine were injected subcutaneously, the first in mazola oil, the second in aqueous solution.

All operations were done under ether anaesthesia.

² We are indebted to the Ciba Company Limited for a generous supply of testosterone propionate.

OBSERVATIONS

1. *The early development of the adrenal gland*

i. *The origin and development of the X-zone.* Three groups of animals (see table 1 for source of material and Appendix 1 for histological details) were studied with the object (a) of establishing the approximate time of the first appearance of the X-zone in our colony and (b) of verifying, if possible, Waring's view that the X-zone is a development from certain cells of the cortical anlage.

TABLE 1

The pre- and early post-natal development of the X-zone.

GROUP	SERIAL NUMBERS ¹	NO. OF ANIMALS	SEX	SOURCE OF MATERIAL
I	D ₋₇ -D ₋₁	11	Not recorded	Adrenals taken from embryos from 13th to 19th day of pregnancy. Material from the 15th, 16th, 17th and 19th days in duplicate
	D ₀ -D ₁₆	18	Males and females	Adrenals from animals 0-16 days of age. Two animals used for 8th-day material
	D ₁₈ -D ₃₀	7	Females only	Adrenals taken from animals every second day from the 18th to 30th day of age
II	X ₁ -X ₈	4	Not recorded	Adrenals studied separately with special stains. Aged 1-3 days.
III	Z ₀ -Z ₁₂	12	Males only	6 animals injected with colchicine and killed 5 (2 animals) and 8 (4 animals) hours later; 6 controls. Aged 13-19 days

¹ Parturition was considered as occurring on the 20th day and is represented as "0" in the animal numbers. D₋₁ is a 19th-day-old embryo, D₁, a 1-day-old mouse; the others are numbered accordingly.

Thirty-six animals were used in the first group which contains both pre- and post-natal material. The adrenals for the pre-natal study were taken from embryos from the thirteenth to the nineteenth day of pregnancy. The pregnant animals were killed and either the whole embryo or a slice containing the glands taken for fixation. Serial sections of both adrenals were made. Eleven pregnant animals in all were used; the

fifteenth, sixteenth, seventeenth and nineteenth days were examined in duplicate. Post-natal material came from twenty-five animals; eighteen pairs of glands were taken from animals from the day of birth to the sixteenth day inclusive (two animals supplied material for the eighth day), and seven pairs were taken from animals every second day from the eighteenth to the thirtieth day. In the series D_0 - D_{16} both males and females were used; in D_{18} - D_{30} females only.

Four animals were studied in the second group, each gland being treated individually so that eight separate glands were examined.

Twelve male animals made up the third group; six were injected with colchicine and six used as controls.

The cortical anlage in the mouse appears in the coelomic epithelium at about 12 days (Waring, '35), the medullary anlage at 13 and the first sign of their union is on the fourteenth. At the fourteenth day the cortical cells are arranged in irregular columns or groups separated by sinuses. The cell walls are absent or indistinct; the cytoplasm is deeply eosinophil; the nuclei are large, ovoid or kidney-shaped and for the most part vesicular, with one or more nucleoli. The sympatho-chromaffin cells are smaller, basophil, and found singly or grouped in nests in the cortical mass, the latter corresponding to the "rosettes" of Pankratz ('31) and Keene and Hewer ('27).

At the sixteenth day of embryonic life a distinct change is noted in the character of the cortical cells from the picture described above. Many assume features characteristic of the future fasciculata; they become cuboidal, more vacuolate and can be differentiated from the darker peripheral cells — the adult glomerulosa. It is at this period that Waring ('35) first noted indications of the formation of his interlocking zone (X-zone of Howard). Some cortical cells do not undergo the above changes but retain the dark eosinophil staining reaction and syncytial arrangement characteristic of the fourteenth day. These cells are found in greatest number in the center of the gland intermingling with, and immediately surrounding,

the sympatho-chromaffin cells. By the day of birth according to Waring they form a layer 1-2 cells deep in most places.

In our tissue the X-zone (interlocking zone of Waring) is not discernible at such an early age (Appendix 1 and fig. 2). It is evident by the fourth day postnatally (fig. 1) although eosinophil cells, singly or in groups, can be seen in this region as early as the day of birth and if these be accepted as the beginning of an X-zone the discrepancy in the appearance of the area in the two groups of experimental animals is not great. Masui and Tamura ('26) found an X-zone appearing in their series from the fifth to fifteenth day of age while Howard ('39) found it differentiating between the fifth and fifteenth days and clearly established by 16-20 days. Beyond this stage, the development of the X-zone in our material (Appendix 1 and 2) is much like that described by others. In the male the zone continues to develop up to about 25-30 days and then begins to degenerate. Degeneration is almost complete by 36 days. Waring found it practically complete by 37 days and Howard ('27) by 38. In the female growth of the zone is maintained well beyond this age.

Waring has no real evidence to support his view that these cells actually derive from the cortical anlage, and simply states that in their staining reaction they possess the "highly eosinophil cytoplasm" characteristic of the cortical substance at the fourteenth day prenatally. From a careful study of our embryonic and early post-natal material we are not prepared to say that these eosinophil cells seen in the neighborhood of the forming medulla are cells from the cortical anlage. All cells of the adrenal alter considerably during this early period and eosinophil cells can be found in any part of the gland.

ii. *The X-zone and reticularis.* In support of his view that the X-zone is transitory in nature Waring points out that in both the male and female mouse a reticular zone appears from the fasciculata that is distinct from the X-zone and can be seen simultaneously with it. Both Howard ('27) and Deanesly ('28) described a ring of dark cells that sometimes

persists outside the fibrous band after degeneration of the X-zone and state that it may be homologous with the zona reticularis of other animals. If such is the case it is distinctly less marked than that of other mammals; Deanesly says that in small adrenals it is poorly developed and inconspicuous, and even Waring (p. 355) states "close examination shows little cytological difference between cells of the zona reticularis and those of the zona fasciculata." In a series of thirty-one immature males, ranging in age from 21–42 days (Appendix 2), i.e. the critical period according to Waring of the degeneration of X-zone and appearance of reticularis we have failed to find a distinctive zone that could be considered as reticularis and agree with Howard ('27) that there is little reason to consider the adult male adrenal as being made up of more than two zones — i.e. a glomerulosa and a fasciculata.

iii. Special fixatives and stains: mitoses. The adrenals from animals recorded in groups II and III, table 1, were examined with the hope of getting further information on the development of the X-zone. Those in group II were taken from mice aged 0–3 days, i.e. just prior to the age when the X-zone becomes clearly discernible in our animals, and fixed and stained with different reagents (see Materials and Methods) in order to determine if these agents would differentiate more clearly, than did haematoxylin and eosin, the developing X-zone cells from the rest of the cortex. No improvement over the routine methods was found.

To study mitotic activity in the adrenal at a period when the X-zone is rapidly developing (13–19 days), six animals (group III) were administered colchicine (Ludford, '36); two received 1 mg. each and were killed 5 hours later; four received 0.1 mg. each and were killed after an interval of 8 hours. In only the first two was the action of colchicine marked and these were used for mitotic counts. Thirty separate high power fields were counted in each animal, fifteen of X-zone and fifteen of fasciculata and glomerulosa. In the first animal thirty-three figures were found in the X-zone, twenty-two in the other zones; in the second animal the counts were forty and

twenty-seven respectively. Thus it would appear that at certain stages of growth mitoses may be more rapid in the X-zone than in the rest of the cortex. Whitehead ('33) who has studied mitotic activity in the mouse adrenal at various ages clearly established the fact that this area has powers of independent proliferation. He found that the rate of division in the X-zone was much the same as that in the periphery; it is possible that when we consider the different sizes of cell in the X-zone and periphery our counts would more closely approximate one another. The rapid growth of the X-zone in the prepuberal mouse is also indicated by the zone of cell crowding that occurs at the junction of the fasciculata and X-zone (and where degeneration of the latter frequently begins). This area of flattened cells suggests a pressure area resulting from the inward growth of cortical cells and an outward growth from the X-zone. Neither of these observations, however, helps in determining the origin of the cells of the X-zone.

Although the object of this paper is to discuss the early development of the adrenal gland only in so far as is deemed necessary to describe the origin and development of the X-zone, two observations were made on our material that may conveniently be recorded at this time. The first deals with the union of the cortical and medullary anlagen; the second with the presence of white blood cells in the adrenal.

iv. The union of cortex and medulla. As recorded above the cortical anlage is seen in the mouse at about the twelfth day of intra-uterine life, the medullary anlage at the thirteenth and the first sign of union at the fourteenth. The sympatho-chromaffin cells and their fibers migrate into the adrenal between the fourteenth and fifteenth day. Waring describes two types of sympatho-chromaffin cell infiltration; one which occurs on the fourteenth day of foetal life and one on the fifteenth. The first type (fourteenth day) consists of infiltration of deeply staining sympatho-chromaffin cells unconnected with nerve fibers; the second type (fifteenth day) consists of migrating cells with nerve fibers. We have been able to find

only the second type of immigration and find it as early as the fourteenth day (fig. 3). In this particular section the nerve fibers deeply penetrating the adrenal, with sympathochromaffin cells at their extremities and along their course are clearly seen; the sympathetic mass from which they came is not seen in the photograph but is observed by tracing back through adjoining sections. Inaba (1891), clearly described and pictured the entrance of nerve fibers and cells into the adrenal on the fourteenth day and does not appear to have seen this first type of migration. Immigration occurring on the fifteenth day is seen in figure 4 which illustrates better the relationship of the invading nerve cells and sympathetic plexus. This migration can be seen in older embryos up to the eighteenth day; a similar observation has been made for the rat (Pankratz, '31). Beyond this period the glands were removed intact and all nervous connections naturally disturbed.

Waring ('35) found only one type of migrating cell, a small dark staining cell with little cytoplasm, similar to what Keene and Hewer ('27) have called neuroblasts in the human gland, but finds three types in the nerve mass applied to the adrenal surface; the small granular migrating cell, large typical ganglion cells, and cells intermediate between these two. We, too, have seen these three types in the sympathetic plexus and are able to substantiate Waring in the view that in the mouse only the small cell migrates. Occasionally cells have been seen on the migratory path, somewhat larger and more irregular than the typical dark migrating cells, but without evidence to the contrary we are inclined to consider them normal variations of the small cell. Keene and Hewer ('27) in the human, and Pankratz ('31) in the rat have found two types of penetrating cells apart from typical ganglion cells; first a small dark cell (their neuroblast) which is abundant, and second a larger cell with a vesicular nucleus. Thus, it is possible that in the mouse other types of cells do migrate but as yet have not been recorded.

Waring points out that the migrating cells change their appearance rapidly after entrance into the gland; they assume basophil cytoplasm and stand out clearly against the background of eosinophil cortical cells. We are able to confirm this observation.

v. Blood cells and leukocytic infiltration. References to the presence of blood cells in the embryonic mouse adrenal are made by Waring ('35) and Inaba (1891), in the rat by Pankratz ('31), and in man by Keene and Hewer ('27). They are seen in all our earlier preparations. In the period 2–8 days post-natally Waring (p. 350), however, mentions the presence of “darkly staining nuclear bodies, scattered throughout the cortex — sometimes more concentrated in one place — sometimes in another. These bodies are very opaque but under intensive illumination they appear to show nuclear figures.” And again (p. 353) in the 9–25 age period “In this area (region of glomerulosa) there are aggregations of black bodies strongly reminiscent of those recorded from the medulla and cortex, between birth and 8 days. Under strong illumination they show some structure, which strongly suggests that they are condensed mitotic figures.”

Areas of leukocyte and lymphocyte infiltration in the medulla and cortex of our mice are found at practically all stages but particularly from the second to the eighth day (Appendix 1): Normablasts too are found in large numbers at this period. The cells are seen singly or more frequently in groups, sometimes in the periphery but generally in the medulla, in the large sinuses and between the cortical cells. Many appear to be in active mitosis and it is frequently difficult to differentiate the mitosis of a blood cell from that of an adrenal cell. We feel that these white and red blood cells may be the bodies seen by Waring. Areas of infiltration are pictured in figures 5 and 12. Two megakaryocytes are pictured in figure 6.

The presence of the cells in such large numbers is difficult to explain. McEuen and Selye ('35) reported areas of infiltrating leukocytes and lymphocytes in tumour-bearing rats,

their absence in normal animals, and suggest that their occurrence in the former may represent a reaction to necrotic processes occurring within the tumours. As far as we are aware our mice were perfectly healthy and tissue decomposition can hardly be advanced to account for their presence in our animals.

2. Regeneration of the X-zone

i. Regeneration following pregnancy. Masui and Tamura ('26), Tamura ('26), Howard ('27) and Deanesly ('28) have all reported rapid involution of the X-zone during pregnancy. Howard ('27) studied this effect in some detail at various ages during gestation and also in the post partum period from within 24 hours of parturition to 14 days following it. Pregnancy was found to precipitate degeneration in the young adult female mouse at an earlier age than it would have occurred normally but otherwise not to influence the gland. Complete disappearance of the X-zone was observed in 5 days; degeneration was most marked in the last third of pregnancy and in the post partum period.

In contradistinction to the numerous reports on the degeneration of the X-zone during pregnancy only one reference was found to its recovery after pregnancy, namely that of Masui and Tamura ('26) in which, unfortunately, no specific data are presented. It occurred to us that lactation might play a role in inhibiting repair of the X-zone in the young adult female and to test this possibility a small group of animals was studied (table 2).

Data have been obtained from twelve animals. The females were placed with males and when pregnant isolated and the date of parturition recorded. Animals P_3 , P_4 , P_6 and P_7 were permitted to nurse their young, the others not, the young being destroyed at birth or on the following day. The left adrenal was removed the day after parturition from all mice except P_6 in which it was removed 3 days later and P_{11} and P_{12} in which it was removed 2 days later. Histological sections

of the left adrenals showed that at parturition the X-zone had either completely degenerated or that traces only remained. Sections of the right adrenals removed after an interval of 12 to 55 days revealed the influence of lactation on X-zone regeneration. No repair had occurred 21 to 26 days post partum in suckling animals; regeneration was good in the majority of non-nursing animals even as early as 12 days following parturition.

TABLE 2

Recovery of X-zone following pregnancy in the young adult female.

ANIMAL NUMBER	AGE AT PARTURI- TION DAYS	DAYS ADRENALS REMOVED AFTER PARTURITION		YOUNG AT PARTURITION	STATE OF X-ZONE	
		Left	Right		Left adrenal	Right adrenal
P	92	1	21	Destroyed	Absent	Occupies $\frac{1}{4}$ - $\frac{1}{2}$ cortex
P ₁	95	1	12	Destroyed	Trace only	Occupies $\frac{1}{4}$ - $\frac{1}{2}$ cortex
P ₂	98	1	45	Destroyed	Absent	Some recovery but less than above two
P ₃	113	1	21	Nursed	Absent	Absent
P ₄	119	1	21	Nursed	Absent	—— ¹
P ₅	60	3	26	Nursed	Absent	Trace only
P ₇	62	1	24	Nursed	Absent	Trace only
P ₈	54	1	50(8) ²	Destroyed	Absent	Small degenerating zone
P ₉	56	1	55	Destroyed	Absent	Occupies $\frac{1}{3}$ - $\frac{1}{2}$ cortex
P ₁₀	57	1	54	Destroyed	Trace only	Occupies $\frac{1}{3}$ - $\frac{1}{2}$ cortex
P ₁₁	64	2	35	Destroyed	Absent	Trace
P ₁₂	64	2	47	Destroyed	Trace only	Occupies $\frac{1}{3}$ - $\frac{1}{2}$ cortex

¹ Right gland lost.

² Fifty days after the first parturition and approximately 8 days pregnant when killed.

P₈ was followed through a second pregnancy. After the first parturition the young were destroyed, the animal isolated for 3 weeks, and then again placed with a male. When approximately 8 days pregnant she was killed. The left gland removed the day following the first pregnancy was devoid of an X-zone; the right removed 50 days later when the animal was in its second pregnancy had a small degenerating zone. Its presence at this time suggests very strongly that regeneration had occurred and that it was now breaking down during the second pregnancy.

The observation of almost complete degeneration of the X-zone at parturition is to be expected but the apparent recovery in these animals which were not allowed to suckle their young is significant. The series is small and recovery did not occur in every case but when considered in the light of the known degeneration during pregnancy it is obvious that repair of the X-zone can occur. Figure 7 pictures the left adrenal of animal P, the day following parturition and figure 8 the right adrenal 20 days later. The presence of a well defined zone in the latter is obvious; in its staining reaction and histological details it appears to be identical with the original X-zone.

TABLE 3

Recovery of the X-zone following testosterone propionate administration in the young male castrated at 22 days of age.

ANIMAL NUMBER	MG. OF TESTOSTERONE PROPIONATE DAILY	INJECTED DAYS ¹	— DAYS ADRENALS REMOVED AFTER LAST INJECTION		CONDITION OF X-ZONE	
			Left	Right	Left	Right
1	0.5	4	1	21	Absent	$\frac{1}{8}$ – $\frac{1}{2}$ cortex
2	0.5	4	1	26	Trace	$\frac{1}{2}$ cortex
3	0.25	4	1	21	Absent	$\frac{1}{8}$ – $\frac{1}{4}$ cortex
4	0.25	4	1	26	$\frac{1}{8}$ – $\frac{1}{4}$ cortex	$\frac{1}{8}$ – $\frac{1}{2}$ cortex
5	—	—	1	21	$\frac{1}{8}$ – $\frac{1}{2}$ cortex	$\frac{1}{8}$ – $\frac{1}{2}$ cortex
6	—	—	21	21	$\frac{1}{2}$ – $\frac{3}{4}$ cortex	$\frac{1}{2}$ – $\frac{3}{4}$ cortex
7	—	—	1	26	$\frac{1}{2}$ cortex	$\frac{1}{2}$ cortex

¹ Injections were started the day following castration.

Thus it is possible that during the early life of the animal the X-zone may show cyclic degeneration with, and repair following, pregnancy. If such did occur it would naturally be restricted because of the onset of the normal process of degeneration (Howard, '27).

ii. *Regeneration following testosterone propionate.* Recovery of the X-zone after administration of testosterone propionate was also studied in the young castrate male. It is well established that castration results in a persistence of the X-zone (Howard, '39), and also that male hormone injection causes a degeneration of the zone (Deanesly and Parkes, '37; Starkey and Schmidt, '38). Table 3 summarizes the results

from seven young male mice, four of which received testosterone propionate for a period of 4 days. Control adrenals (left) were removed the day following the last injection. The right adrenals were examined after a recovery period of 21 to 26 days. The effect of the steroid on the control adrenal and the subsequent regeneration is illustrated in figures 9 and 10. Although in some instances the regenerated zone may have been somewhat smaller than the primary X-zone no distinction could be noted in the type of cell and staining reaction of the two areas.

In this connection it is interesting to observe that the reticularis of a control series of rats was not influenced by 8 to 17.5 mg. of testosterone propionate given over a period of 10–16 days (fig. 11) whereas 0.25 mg. daily for 4 days caused complete degeneration of the mouse X-zone. This suggests that the X-zone is functionally different from the reticularis of other mammals.

No references to repair of the X-zone following its destruction by steroids could be found. Selye ('40) in speaking of the effects of testosterone propionate on the adrenal says the "actions are still detectable months after the injections are discontinued." But no details of the experiments are given.

DISCUSSION AND CONCLUSIONS

The physiological significance of the X-zone and its relation to the reticularis of other mammals has not been adequately explained. Waring ('35) considers that it is embryonic in origin, transitory in nature, and homologous with the boundary zone of man (Elliott and Armour, '11). Although the boundary zone in man is transitory in nature its origin has not been definitely settled. It may be pertinent at this time to weigh more carefully the evidence for and against the view that the X-zone is homologous with the transient area of man and that it possesses properties distinct from those of adult cortical cells.

i. Origin. The experimental findings of Waring have already been discussed as well as our own failure to substantiate his claims. Other workers, however, from the school of zoology, University of Liverpool, have evidence of similar areas, transitory in nature, in the adrenal glands of other mammals; e.g. Davies ('37) pictured an inner zone in the foetal cat adrenal which disappears in early post natal life and thought it homologous with the mouse X-zone. Roaf ('35) for the rabbit states — "the innermost cortical zone is interlocked with the medulla and from available embryological evidence it appears to represent the remains of the foetal cortex after the reticular, fasciculata and glomerulosa zones have differentiated." Unfortunately his series of embryos was small and Davies could not trace the origin of the zone in her cat material. Further, as Howard ('27) observed, if the X-zone is homologous with the boundary zone of man there is a marked discrepancy in the age at which they appear.

ii. Mitoses. The presence of considerable mitotic activity in this region might argue for an independent origin of the X-zone. However the fact must not be overlooked that mitoses take place in all parts of the adrenal gland and the increased activity might well occur in X-zone cells after they have formed from the fasciculata.

iii. Regeneration following degeneration. Some confusion exists in the literature as to whether or not the regenerated X-zone is similar to the primary one. Deanesly ('28) stated that after castration "an area develops which eventually resembles the X-zone in the young female in all respects — but in animals castrated after sexual maturity growth in the adrenal cortex appears to cease before the condition characteristic of the young female is reached." Callow and Deanesly ('35) say that the "zona reticularis — reappears after castration in the mouse." Howard in various papers ('27, '38 and '39) discusses the question and although she states that the primary and secondary X-zones have a "some-

what different structural organization'' ('39) yet she considers them so alike that "this is definite evidence that the X-zone is a differentiation from the cortex rather than a fundamentally different tissue of other origin."

Our evidence for the reappearance of an X-zone following pregnancy and testosterone propionate administration supports the view that the development of the new X-zone is identical in every way with the primary zone.

Although we feel that the X-zone can develop from the fasciculata yet we consider this area as distinct from the reticularis of other animals. Considerably greater quantities of testosterone propionate than were necessary to cause complete degeneration of the X-zone in the mouse had no effect on the reticularis of the rat.

SUMMARY

1. The embryonic and early post-natal development of the mouse adrenal has been studied. No conclusive evidence could be found to support the view that the X-zone is a transitory development from the cortical anlage.

2. Migration of nerve fibers and cells into the adrenal gland have been described on the fourteenth and fifteenth days of intra-uterine life.

3. Leukocyte and lymphocyte cell infiltration in the early post-natal life of the adrenal is described.

4. Regeneration of the X-zone following pregnancy and testosterone propionate administration has been found to occur. The new zone is thought to be identical with the primary X-zone.

5. Evidence for and against the view that the X-zone is a transitory development from the original embryonic cortical cells is summarized. It is felt that the X-zone is derived from the fasciculata.

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APPENDIX 1

To establish age at which X-zone first become discernible in the mouse.

NO. OF MOUSE AND AGE IN DAYS ¹	SEX	REMARKS	MITOSES	BLOOD CELLS
D ₋₇		Cortical cells appearing. Much as in D ₋₆		
D ₋₆		Cortical cells large and eosinophilic. Nuclei large, ovoid, and vesicular. Cell walls present but not distinct. S.C. scattered and clumped at terminations of nerve fibers	In body of cortex	Embryonic R.B.C.
D ₋₅		As in D ₋₆ . Cells in irregular columns	In periphery and body of cortex	Embryonic R.B.C.
D ₋₄		Cortical cell walls distinct. Clumping of eosinophil cells in center. Fasciculata appearing	In periphery and body of cortex	Embryonic R.B.C.
D ₋₃		Cortical cell walls distinct. Nuclei rounder. Some darker cortical cells in center of gland	In body of cortex	Few embryonic R.B.C.
D ₋₂		Much as above. Columns of cells more regular	In body of cortex	Few embryonic R.B.C.
D ₋₁		Cell walls distinct. Nuclei rounder. S.C. confined to center. Cell columns more regular	In medulla and periphery	Numerous embryonic R.B.C.
D ₀		Nuclei round. Intermingling of medullary and cortical cells. Individual dark cells — possible X-zone cells	Mainly in periphery	Few R.B.Cs.
D ₁		As in D ₀	Mainly in periphery	Few R.B.Cs.
D ₂		As in D ₁	In medulla and cortex	Many R. and W.B.Cs.
D ₃	F	As in D ₂ but more advanced	Everywhere	Many R. and W.B.Cs.
D ₄	F	X-zone discernible. Cell walls distinct	Few in cortex	Many R. and W.B.Cs.
D ₅	F	X-zone present. Cell walls distinct	Rare	Few
D ₆	M	X-zone present. Cell walls distinct	Cortex and medulla	Many R. and W.B.Cs.
D ₇	F	X-zone 5-6 cells deep	Many in X-zone and periphery	Few R. and W.B.Cs.
D ₈	M	X-zone 5-6 cells deep	In X-zone	Many W.B.Cs.
D ₉		X-zone 4-6 cells deep	In body of cortex and X-zone	Many R. and W.B.Cs.
D ₁₀		X-zone many cells deep	In X-zone	Few blood cells
D ₁₁	M	X-zone 3-6 cells deep	Medulla, cortex and especially X-zone	Few blood cells
D ₁₂	M	X-zone 5-10 cells deep	Few scattered	
D ₁₃	F	X-zone 4-8 cells deep	Few in periphery and X-zone	
D ₁₄	F	X-zone $\frac{1}{3}$ of cortex. Pressure area at border of X-zone and fasciculata	Scattered	
D ₁₅	M	X-zone $\frac{1}{3}$ of cortex. Pressure area at border of X-zone and fasciculata but not as marked as in D ₁₄	Fewer	
D ₁₆	F	X-zone $\frac{1}{3}$ - $\frac{1}{2}$ of cortex. Pressure area at border of X-zone and fasciculata	Scattered	
D ₁₉	F	X-zone $\frac{1}{3}$ of cortex. Pressure area	Few in cortex	
D ₂₀	F	X-zone $\frac{1}{3}$ of cortex. Pressure area	Few in cortex	
D ₂₂	F	X-zone $\frac{1}{3}$ of cortex. Pressure area	Few in cortex	
D ₂₄	F	X-zone $\frac{1}{3}$ of cortex. Pressure area	Few in cortex	
D ₂₆	F	X-zone $\frac{1}{3}$ - $\frac{1}{2}$ of cortex. Pressure area	Few in cortex	
D ₂₈	F	X-zone $\frac{1}{3}$ of cortex. Pressure area	Few in cortex	
D ₃₀	F	X-zone 3-4 cells deep. Pressure area	Frequent in X-zone	

¹0 = birth at 20 days; D₋₁ = adrenal from 19-day-old embryo; D₁ = adrenal from 1-day-old mouse. Other numbers accordingly.
S.C. = Sympatho-chromaffin cells. R.B.C. = Red blood cells. W.B.C. = White blood cells.

APPENDIX 2

ANIMAL NUMBER	AGE IN DAYS	X-ZONE
1	21	Well developed X-zone
2	26	X-zone $\frac{1}{4}$ cortex
3	26	X-zone $\frac{1}{4}$ - $\frac{1}{3}$ cortex
4	27	X-zone $\frac{1}{4}$ - $\frac{1}{3}$ cortex
5	27	X-zone $\frac{1}{4}$ cortex
6	27	X-zone $\frac{1}{4}$ cortex
7	27	X-zone $\frac{1}{4}$ cortex
8	27	X-zone $\frac{1}{4}$ cortex
9	28	No X-zone
10	26	X-zone $\frac{1}{3}$ - $\frac{1}{2}$ cortex
11	26	X-zone $\frac{1}{2}$ cortex
12	30	X-zone $\frac{1}{2}$ cortex
13	31	Small X-zone 2-3 cells deep
14	31	X-zone $\frac{1}{3}$ - $\frac{1}{4}$ cortex
15	32	No X-zone
16	32	X-zone $\frac{1}{4}$ cortex
17	33	X-zone $\frac{1}{3}$ cortex
18	34	X-zone 4-6 cells deep
19	36	X-zone $\frac{1}{3}$ cortex
20	36	No X-zone
21	36	No X-zone
22	36	No X-zone
23	36	No X-zone
24	36	No X-zone
25	36	No X-zone
26	38	Trace only
27	40	No X-zone
28	41	No X-zone
29	41	No X-zone
30	41	No X-zone
31	42	X-zone, trace only

PLATE 1

EXPLANATION OF FIGURES

- 1 Adrenal from 4-day-old mouse showing beginning of X-zone (area marked). Note clumps of leukocytes and lymphocytes in medullary area. $\times 250$.
- 2 Adrenal from mouse 1 day old. No X-zone visible. $\times 100$.
- 3 Adrenal from embryo at the fourteenth prenatal day showing migration of nerve fibers (N.F.) and cells (C). Note area of dark sympatho-chromaffin cells in middle of section. $\times 240$.
- 4 Adrenal from 15-day-old embryo showing sympathetic mass (S) and nerve fibers (N.F.) penetrating gland. $\times 108$.

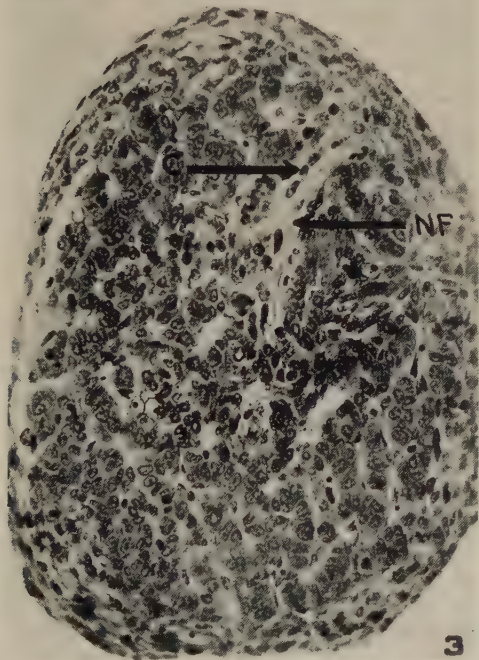
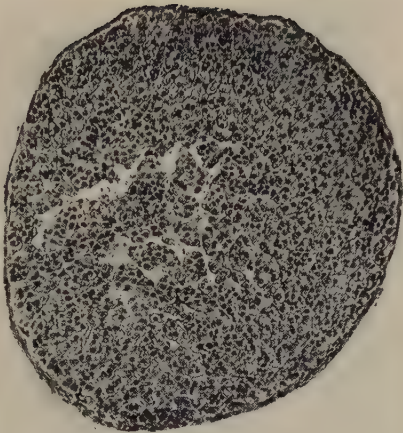
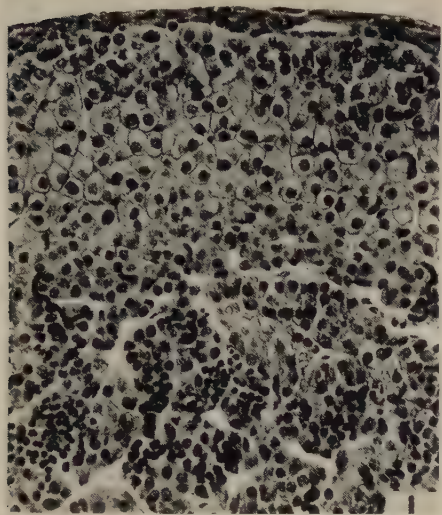


PLATE 2

EXPLANATION OF FIGURES

- 5 Adrenal from 4-day-old mouse showing beginning of X-zone. Note clumps of leukocytes and lymphocytes in central part of gland. $\times 106$.
- 6 Adrenal from 8-day-old mouse showing two megakaryocytes indicated by arrows. $\times 340$.
- 7 Left adrenal from mouse P removed day following parturition. Note absence of X-zone. $\times 108$.
- 8 Right adrenal from mouse P removed 21 days after parturition. X-zone has redeveloped. $\times 84$.

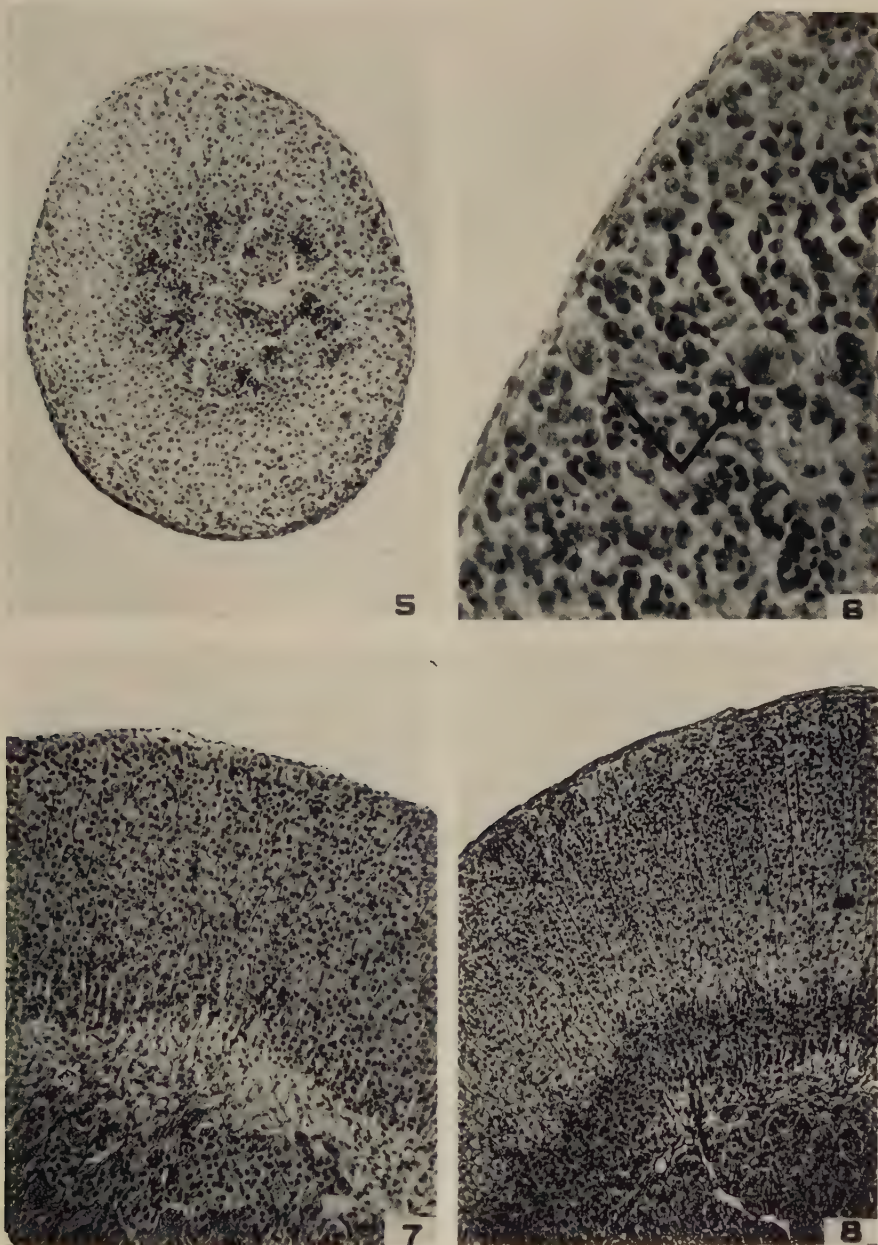
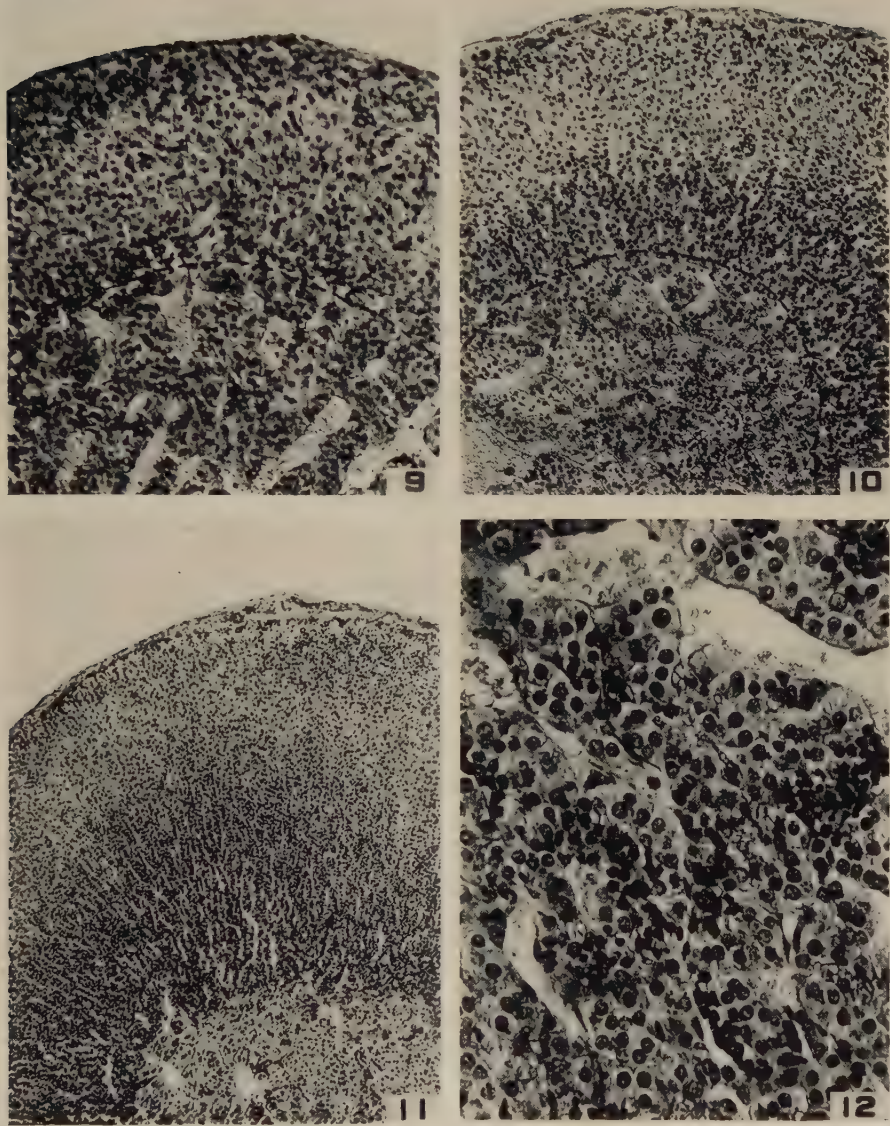


PLATE 3

EXPLANATION OF FIGURES

- 9 Left adrenal removed from mouse no. 1 after four daily injections of 0.5 mg. of testosterone propionate. X-zone absent. $\times 112$.
- 10 Right adrenal removed from same mouse (no. 1) 20 days later. X-zone has redeveloped. $\times 112$.
- 11 Adrenal removed from rat after receiving 8 mg. of testosterone propionate over a period of 16 days. Reticularis present. $\times 63$.
- 12 Portion of adrenal from 5-day-old mouse showing clumps of dark leukocytes and lymphocytes. $\times 315$.



THE MOUSE ADRENAL

II. THE ACTION OF CERTAIN HORMONAL SUBSTANCES ON THE ADRENAL GLAND OF THE MOUSE WITH PARTICULAR REFERENCE TO THEIR ACTION ON THE X-ZONE ¹

M. K. McPHAIL AND H. C. READ

Department of Pharmacology, Dalhousie University, Halifax, N. S.

TWO PLATES (NINE FIGURES)

INTRODUCTION

The present paper deals with the action of certain hormonal substances on the mouse adrenal, particularly with the changes brought about in the X-zone. Studies of this nature have previously been made. Deanesly and Parkes ('37); Cramer and Horning ('37 a); Starkey and Schmidt ('38); Howard ('40) and Selye ('40) have all reported rapid disappearance of the X-zone in the male and female mouse following administration of testosterone and other androgens.

The effect of oestrogens on the X-zone is by no means so decisive. In the male, Burrows ('36) found that local application to the skin for long periods brought about first, a reappearance of the X-zone and then extensive degenerative changes consisting of the formation of "large rounded lipoid-like masses." Cramer and Horning ('37 a), report no action on the X-zone of the female; ('37 b), the appearance of "brown degeneration" in both sexes following cutaneous application of oestrogens. The "brown degeneration" is found in isolated masses or as a continuous band between the cortex and medulla and is described as "necrotic tissue impregnated

¹ We are grateful to the Banting Research Foundation for a personal grant to one of us (H.C.R.).

with lipoid." Lacassagne and Raynaud ('37) state that involution of the X-zone is not modified by oestrogens but describe reduction in all the zones of the cortex. Danner ('38) found great degenerative changes in the zona fasciculata and reticularis but not in the glomerulosa after oral treatment of 4-16 months' duration. Martin ('30-'31), giving few experimental details, states that prolonged oestrin treatment caused total degeneration of the X-zone in immature castrated males and normal and spayed females. In immature non-castrated males oestrin caused a persistence of the X-zone and in non-castrated adult males a reappearance of the X-zone. Hall ('40) reported that long term injections (87-105 days) of oestradiol esters resulted in hyperaemia and degeneration of the cells of the reticular zone of the rat. In extreme cases the reticularis completely disappeared with islets of tissue frequently persisting between the cortex and medulla. These may be similar to the "brown degeneration" described by Cramer and Horning ('37 b) in the mouse. In summary, although there is no such unanimity in the experimental findings as with the androgens, yet all workers, with the exception of Martin, have described cortical reduction or degeneration following oestrogen therapy.

Few studies have been made of the action of gonadotrophic substances on the X-zone. Martin ('30-'31) found that injections of the luteinizing hormone of the anterior pituitary for short or long periods had no effect on the zone; prolonged injections of gonad stimulating hormone of anterior pituitary increased the size of the zone in the immature normal and spayed female and caused its persistence in the immature normal male. Cramer and Horning ('37 a) found pituitary hormones without action on the X-zone.

Preston ('28) and Gersh and Grollman ('39) report hypertrophy and hyperplasia from administration of thyroid extracts and Gersh and Grollman ('39) atrophy from adrenal cortical hormones.

Howard ('40) reported no action from desoxycorticosterone acetate; Gersh and Grollman ('39) and Selye ('40) involution

of the X-zone, although the zone had not completely disappeared in all the animals studied by Gersh and Grollman.

Howard and Gengradom ('40) described a slight reduction, but not disappearance, of the X-zone following large doses of progesterone; Selye ('40) found it to disappear.

Our report deals with the effect of testosterone propionate² oestrone, progesterone, anhydro-hydroxy-progesterone, desoxycorticosterone acetate, stilboestrol, and urinary A.P.L. and P.M.S. hormones on the X-zone, and the repair of the latter following testosterone propionate and A.P.L.

MATERIALS AND METHODS

The mice used in the present report came from a mixed colony which has been maintained in the department for the past 2 years. Routine histological procedures were used in the preparation of material for examination. All injections were made subcutaneously; the steroids in oil, the gonadotrophins in aqueous solution. The A.P.L. and P.M.S. were stored as dry powders in a desiccator. Material for injection was prepared daily by diluting from concentrated solutions made up approximately once a week and kept, except during the preparation of the weaker solutions, frozen in the ice-box.

OBSERVATIONS

1. A.P.L. and P.M.S. hormones

One hundred and fourteen animals (male and female, gonadectomized and normal, immature and adult) were used in the study of the action of A.P.L. and P.M.S. gonadotrophins on the X-zone. These agents caused involution of the zone in the young intact animal similar in kind to that obtained from testosterone although somewhat slower in

² We are indebted to the Ciba Co. Ltd., for a generous supply of testosterone propionate; to the Schering Corp., for the desoxycorticosterone acetate and anhydro-hydroxy-progesterone; to Dr. A. S. Cook of Ayerst, McKenna and Harrison for the urinary A.P.L. and P.M.S. hormones; to Merck and Co. Inc., for the stilboestrol; and to Dr. R. D. H. Heard of the Department of Biochemistry for the oestrone and progesterone.

development (figs. 1-4). The action is mediated through the gonads; in the castrate male and spayed female no effect is obtained. Results of some of the experimental work are given in table 1.

TABLE 1

Action of A.P.L. and P.M.S. on the X-zone of the adrenal of the immature male and female mouse. Animals castrated day of first injection; killed day following last injection. Litter mates used as controls.

TREATMENT AND AMOUNT DAILY	PREPA- RATION	NO. OF ANI- MALS	SEX	AGE WHEN EXP. STARTED (DAYS)	NO. OF DAYS OF TREAT- MENT	X-ZONE
A.P.L. 11 I.U.	Normal	5	M	16	10	Absent
A.P.L. 11 I.U.	Castrate	4	M	16	10	Well developed
Controls	Normal	2	M	16	..	Well developed
A.P.L. 10 I.U.	Normal	5	F	20	10	Absent (3) trace (1) Present (1)
A.P.L. 10 I.U.	Castrate	5	F	20	10	Well developed
Controls	Normal	5	F	20	..	Well developed
P.M.S. 11 I.U.	Normal	4	M	16	10	Absent
P.M.S. 11 I.U.	Castrate	3	M	16	10	Well developed
Controls	Normal	1	M	16	..	Well developed
P.M.S. 11 I.U.	Normal	3	F	20	10	Absent or trace
P.M.S. 11 I.U.	Castrate	3	F	20	10	Large X-zone
Controls	Normal	2	F	20	..	Large X-zone
P.M.S. 11 I.U.	Normal	3	F	20	5, 10, 15 20, 20, 20	Present only at 5 days

In the adult male mouse an X-zone is found only after castration and in this animal A.P.L. and P.M.S. are without effect. In eight young adult females injected for 6-24 days with 10 I.U. of A.P.L. daily, X-zone degeneration was greater than in three controls. Caution must be exercised in interpreting the degree of degeneration in the young female as vacuolization of this area is so very variable. However, considered along with the positive results in the immature male and female and the rapid degeneration during pregnancy it would be surprising if A.P.L. and P.M.S. did not act in this manner.

Repair of the X-zone following degeneration caused by A.P.L. was studied in ten young females aged 24 days when the experiment was started. In this series the left adrenals

were removed at various periods during treatment and the right adrenals 3–21 days after termination of injections. Thus each animal served to supply data on (a) the amount of gonadotrophin necessary to cause involution of the X-zone and (b) the time required for repair following its dissolution. This scheme was adopted as it has been repeatedly shown that the histological picture of the two adrenals is identical at all phases of the animal's life. The data is too limited to give anything in the way of quantitative information. However, in some animals degeneration of the X-zone was complete in 5 days, in others a small band of tissue persisted after 9 days; repair was good 12–21 days following termination of injections.

2. Pregnancy urine

Three normal and two ovariectomized 21-day-old mice were injected with human pregnancy urine. In two normal females, one injected for 10 days, the other for 15, the X-zone was absent, in the third, injected for 7 days a small X-zone was present. The X-zone was well developed in the two castrates injected for 7 and 15 days.

3. Oestrone and stilboestrol

The most striking effect of oestrone and stilboestrol in the young female was a swelling and vacuolization of the fasciculata immediately peripheral to and frequently involving the X-zone (table 2). In many glands, however, the X-zone was large and apparently little influenced by treatment, while surrounding it was the ring of degeneration which for convenience we have called the "ring effect" (figs. 5 and 8). In the degenerated area the cells swell up, run together and lose their normal staining reaction. This effect is not specific to oestrone and stilboestrol; it has been pictured before and we have seen it occasionally in our A.P.L. and P.M.S. treated animals as well as in those receiving both oestrone and progesterone, but we find it more consistently following these agents than after other hormones. Morrell and Hart ('41)

have described swelling and vacuolization in the fasciculata of the rat after stilboestrol administration. Results from treated and control mice are given in table 2. In addition, two adult females were injected with oestrone, one, with 5000 I.U. daily for 9 days, another with 5000 I.U. daily for 14 days and 1000 I.U. for 4 days. In the first the ring of degeneration was beginning, in the second it was marked. A control did not show this effect.

TABLE 2

The effect of oestrone, stilboestrol, and oestrone and progesterone on the X-zone of the female mouse.

TREATMENT	AMT. DAILY	DURATION OF TREATMENT DAYS	NO. OF ANI-MALS	AGE IN DAYS WHEN STARTED	RESULT
Oestrone	5000 I.U.	2	4	35	"Ring effect" good in
	1000 I.U.	13			3, poor in 1
Controls		..	4	35	Absent
Oestrone	5000 I.U.	3	4	107	"Ring effect" in all
Progesterone	0.5 mg.	5-7			
Controls		..	4	107	Absent
Stilboestrol	0.1 mg.	15-21	15 ¹	26-62	"Ring effect" good in
					8, present in 5, absent
					in 2
Controls		..	6	38-62	Absent in 4, present in 2

¹ Five ovariectomized.

In another experiment eleven males were injected with 250-5000 I.U. daily for periods of 8-15 days with the object of studying the fascicular degeneration in the absence of the X-zone. Vacuolization occurred in two animals only (fig. 9) although a number of our animals either died or were sickly when killed as a result of the high oestrone dosage. Why the reaction occurred so readily in the animals reported in table 2 and not in the males is not known. It may be that the "ring effect" is really the beginning of X-zone degeneration; however, as indicated above in the female, it appeared to involve the fasciculata rather than the X-zone. In several glands there was an increased eosinophil reaction of the fascicular cells much like that seen in early post mortem decay.

In contradistinction to Martin's and Burrow's reports we found no X-zone reappearing in these injected males.

In five immature females treated with oestrone, 1000 I.U. daily for 10 days, the X-zone had degenerated. The left adrenals served as controls. In the only animal examined there was good regeneration of the X-zone 20 days after cessation of treatment.

TABLE 3

To determine the action of oestrone on the juxta-medullary cortex in the absence of the X-zone.

NO. OF ADRENAL ¹	DURATION OF TREATMENT		DEVELOPMENT OF X-ZONE ²
	A.P.L. 10 I.U. daily	Oestrone 1000 I.U. daily	
L ₁	..	..	++++
L ₂	..	..	++++
L ₃	9	..	0
L ₄	9	6	0
L ₅	9	6	+
L ₆	9	12	++
R ₁	9	0	0
R ₂	9	12	+
R ₃	9	6	+
R ₄	9	12	+±
R ₅	9	18	++
R ₆	9	18	++++±

¹ L = left adrenal, R = right adrenal.

² Size of the X-zone is represented by + signs; a large zone occupying half of cortex is represented by +++++, no zone by 0, and intermediate stages accordingly.

4. Oestrone following A.P.L.

It was hoped to study further the degeneration of the juxta-medullary area by oestrone in animals after the X-zone had been destroyed by A.P.L. Ten I.U. daily of A.P.L. were first administered and then 1000 I.U. of oestrone, to six females aged 24 days when injections were started; the adrenals were studied individually so that twelve records were obtained (table 3). In these animals not only was there no fascicular degeneration but repair of the X-zone took place during

oestrone administration and in some it appeared even to be accelerated.

Thus with the exception of the two males reported above we were unsuccessful in our attempts to obtain fascicular degeneration in the absence of the X-zone.

No specific studies were made on regeneration of the adrenals following oestrone administration other than the single animal mentioned above.

5. Testosterone propionate

There are numerous reports (compare above) on the effectiveness of testosterone and its esters in causing total degeneration of the X-zone and our results are in complete accord with these. In young castrate males an injection of 0.5 mg. of testosterone propionate for 3 days, or 0.25 mg. for 4 days, is often sufficient to bring about a complete disappearance of the X-zone. Starkey and Schmidt ('38) report total disappearance in 6 days from a single injection of 0.4 mg. On the other hand we have observed in adult rats, castrate and normal, that 8-17.5 mg. of testosterone propionate given over 7-20 days has no effect on the reticularis.

There are few references to regeneration of the X-zone after its destruction by steroids. Selye ('40) found testosterone lesions still detectable months after injections were discontinued but gives no experimental data. We followed repair of the X-zone in a group of nine young castrates after its destruction by testosterone propionate. Although little regeneration had occurred by 15 days it was quite good 20 and 25 days after discontinuance of treatment.

6. Progesterone and anhydro-hydroxy-progesterone

Three female mice, 20 days of age, were injected with 0.5 mg. of progesterone for 10 days (one animal) and 13 days (two animals). Left adrenals were used as controls. Although there was slight suppression of development, the X-zone was present in all. Four males, castrated at 22 days and injected

at 23 days, were given 0.5 mg. daily for 10 days (two animals) and 15 days (two animals). Three castrate males served as controls; there was no significant difference between the X-zones of the treated and control animals.

Anhydro-hydroxy-progesterone was found to exert more action on the X-zone than progesterone. Of six young female mice, aged 29 days when injections were started, three were injected for 11 days and three for 16 days, with 0.5 mg. of the compound. In two animals the X-zone was almost completely degenerated, in the others it was considerably reduced.

7. *Desoxycorticosterone, acetate*

Our experience with desoxycorticosterone acetate is limited. Three castrated males and six normal females were injected for periods of 5–15 days. In the males a reduction but not disappearance of the X-zone resulted from 0.2 mg. daily for 5 days; in the females 1 mg. daily for 15 days caused involution of the area in most, but not all, animals. Selye ('40) found it to disappear in young castrate male and normal female mice, but he used considerably larger amounts, viz., 3 mg. daily for 20 days. Frequently we found a change in the character of the X-zone, the sinusoidal arrangement being replaced by a condensation of cells such as described by Clausen ('40) in the reticularis of the rat after large doses of progesterone.

DISCUSSION

The finding that A.P.L. and P.M.S. gonadotrophins cause degeneration of the X-zone in the intact male and female mouse offers a plausible explanation for the rapid involution that occurs in this area during pregnancy, i.e. at a time when the system is flooded with gonadotrophic hormones. What the inhibitory substance is, is not known. In view of the capacity of testosterone to cause rapid X-zone involution it is possible that the gonadotrophins stimulate the testes and ovaries to release male hormone and that this is the effective agent. Thus in the female, one must assume either that the ovaries release androgen or that the action is mediated by some other

substance. That the ovaries can exert an androgenic action is well known (Hill, '41). A possible objection to this view is the observation of Hain ('39) that during pregnancy in the human androgen excretion is lowered. The force of this criticism however, is lessened when it is remembered that conditions might be different in the mouse; further there is no proof that androgen is the inhibitory agent.

The sensitivity of the X-zone to hormone injection may be due in part to its receiving the poorest blood supply of any part of the adrenal (Gersh and Grollman, '41). This however would not account for the different response obtained from oestrogen and androgen administration. As our oestrone animals had received very large doses and were obviously ill it occurred to us that the action might be a non-specific one, and with this in mind we examined the adrenals of animals (a) exposed to prolonged chloroform anaesthesia, and (b) during post mortem degeneration. Twelve animals were examined 0-72 hours after exposure to 3 hours (six adrenals) and 6 hours (six adrenals) of chloroform. Although a "ring effect" was found in one adrenal there was no general similarity between the toxic effect of chloroform and the action of oestrone. Post mortem degeneration was studied in fourteen animals from 2-70 hours after death. In two adrenals the picture (fig. 7) of degeneration was reminiscent of the effect of oestrone but again there was no close resemblance between the oestrone action and post mortem changes. The last experiment, however, does show that the X-zone is one of the first parts of the cortex to disintegrate. Oestrone and stilboestrol may produce "exhaustion degeneration" of the fasciculata — i.e. an overproduction of the cortical hormones to the stage of adrenal insufficiency; such an explanation would account for the death and condition of our mice treated with the large doses of oestrone. This action could be either direct on the adrenal or by an inhibition of secretion of pituitary adrenocorticotrophic hormone. Although such a view would account for our ring of degeneration in the young female it would hardly account for the degeneration of the X-zone in the normal immature female on one hand, and the inability of

oestrone to prevent the regeneration of X-zone after A.P.L. destruction on the other. No explanation can be given for these divergent and conflicting results. The majority of workers (compare above), with the exception of Martin, do not report X-zone destruction from oestrone therapy.

The difference between our results with progesterone and desoxycorticosterone acetate and those of Selye ('40) can probably be adequately accounted for by the much larger amounts used by the latter worker.

SUMMARY AND CONCLUSIONS

1. A.P.L. and P.M.S. hormones cause degeneration of the X-zone of young intact male and female mice. They act through the gonads.

2. Oestrone may cause X-zone degeneration but is more prone to produce vacuolization of the inner part of the fasciculata.

3. Progesterone in the amounts used had little effect on the X-zone; anhydro-hydroxy-progesterone and desoxycorticosterone somewhat more, but neither in the doses used, completely destroyed the zone.

4. Regeneration of the X-zone has been studied following destruction by urinary A.P.L. and testosterone.

5. Post mortem degeneration of the adrenal and its reaction to prolonged chloroform anaesthesia were studied with the view of shedding light on the oestrone reaction.

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PLATE 1

EXPLANATION OF FIGURES

- 1 Adrenal from normal 30-day-old mouse with X-zone occupying about one-half of cortex. $\times 95$.
- 2 Littermate after receiving A.P.L., 10 I.U. daily for 10 days. Note complete absence of X-zone. $\times 95$.
- 3 Adrenal from a 30-day-old mouse with well developed X-zone. $\times 95$.
- 4 Littermate following P.M.S. administration, 11 I.U. daily for 10 days. Adrenal contains only a small band of X-zone tissue. $\times 95$.

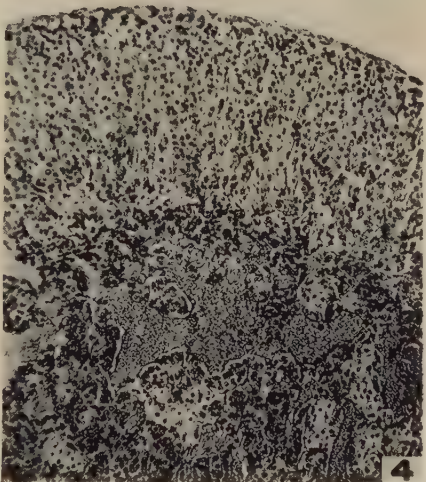
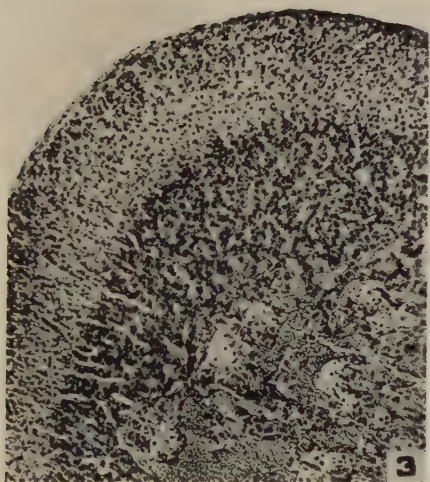
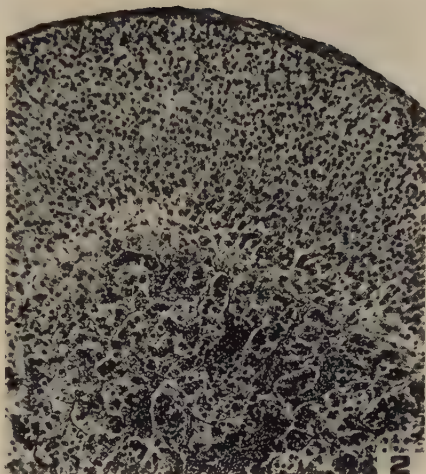
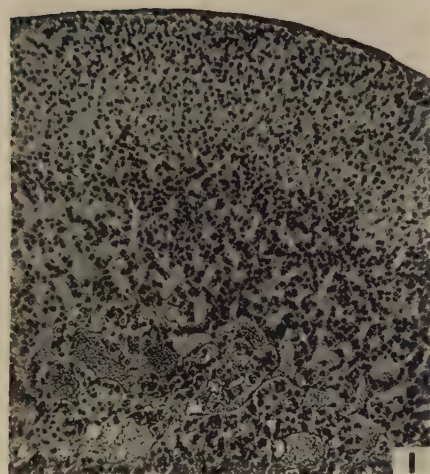
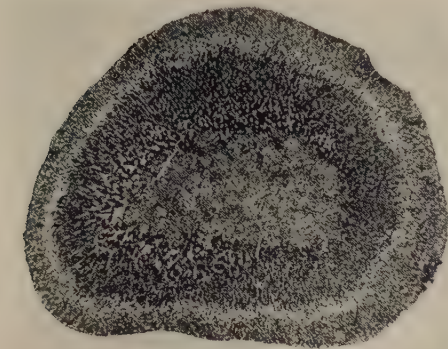


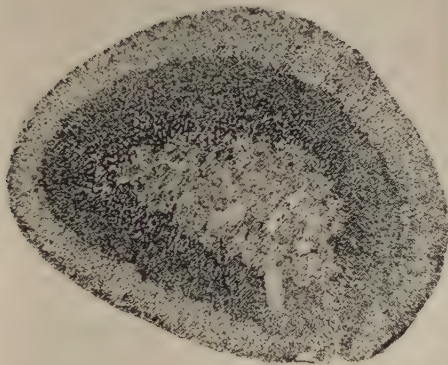
PLATE 2

EXPLANATION OF FIGURES

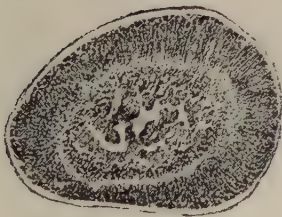
- 5 Ring of degeneration in adrenal from a female mouse receiving 0.1 mg. stilboestrol daily for 21 days. $\times 30$.
- 6 Control littermate of stilboestrol treated animal. "Ring effect" absent. $\times 30$.
- 7 Ring of degeneration in adrenal from mouse 48 hours after death. All cells are degenerate. $\times 30$.
- 8 Adrenal, showing ring of degeneration in cortex, from female mouse following administration of 5000 I.U. oestrone for 3 days and then 0.5 mg. progesterone for 7 days. $\times 70$.
- 9 Fascicular degeneration in male mouse after injection of 5000 I.U. oestrone daily for 15 days. $\times 70$.



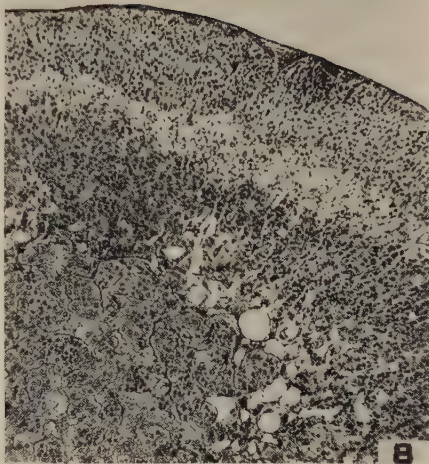
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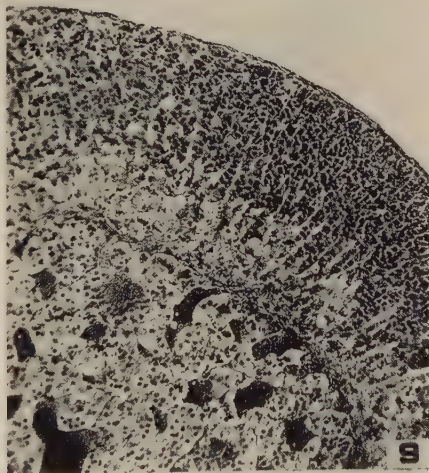
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THE EFFECT OF PERFUSION WITH SODIUM CITRATE ON THE CONTENT AND DISTRIBUTION OF THE MINERALS IN VARIOUS CELLS OF THE CAT AS SHOWN BY ELECTRON MICROSCOPY AND MICROINCINERATION ¹

ALBERT I. LANSING AND GORDON H. SCOTT ²

*Department of Anatomy, Washington University School of Medicine,
Saint Louis, Missouri*

FIVE FIGURES

Hastings and McLean ('34) and McLean and Hastings ('35) have shown that "when calcium and citrate are present in solution, a part of the calcium present is bound in a complex, negatively charged ion." It was also shown that in solutions containing calcium, citrate and protein, the calcium binding power of the citrate exceeds that of the protein. This action of citrate solutions was demonstrated by Mazia and Clark ('36) in their experiments on *Elodea* cells.

The present experiments were designed to determine by cytochemical methods the effect of sodium citrate on the mineral content and distribution in some cells of the cat. It was hoped that a technique for altering the calcium content of the various intracellular structures could be established, and also that some specific information could be obtained as to the identity of some of the ash granules in microincinerated preparations. While it is generally believed that the blue ash in such preparations represents either sodium or potassium or both and that the white ash represents calcium or magnesium or both (Scott, '33), no critical tests have been made to determine the identity of these ash granules.

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² Present address, Department of Anatomy, The University of Southern California School of Medicine, Los Angeles.

PREPARATION OF THE MATERIAL

Adult cats were anaesthetized with ether and small samples of liver, kidney, duodenum, rectus abdominis muscle and femoral nerve were removed for cytochemical analysis. Samples were fixed in absolute alcohol-formalin (9 parts to 1 part) and one set of tissues was prepared by the frozen dehydration technique described by Scott and Williams ('36) and Scott ('36). This latter material was frozen in iso-pentane cooled to liquid air temperature prepared and dehydrated at -63°C . in a cryostat. Material prepared by this method is valuable in that the mineral content and distribution is more faithfully preserved than by alcohol-formalin fixation, which nevertheless, preserves the intracellular distribution of the minerals remarkably well.

Immediately after the removal of the control samples of tissues, a cannula was inserted into the descending aorta and the right ventricle was cut open. The cats were perfused with 500 cc. of 0.95% sodium citrate which was maintained at body temperature. Examination of the various organs was made at this time to ascertain whether or not the perfusion fluid had penetrated thoroughly (as indicated by the presence or absence of blood in the smaller blood vessels). Samples of the previously listed organs were removed and fixed; some were fixed with alcohol-formalin and one set of tissues was frozen in cooled iso-pentane. The alcohol-formalin material was dehydrated in alcohol, cleared in xylol, imbedded in paraffin and sectioned at 4 micra. The frozen material, after dehydration in the cryostat, was imbedded in paraffin in vacuo and sectioned at 4 micra.

Sections of the material fixed by both methods were incinerated according to the procedure established by Scott ('36). The incinerated sections were examined by means of the dark-field microscope (cardioid condenser). Other sections were prepared for examination with the electron microscope the details of which procedure were described by Scott and Packer ('39 a and b). Briefly, the sections were placed on a nickel cathode which had been coated with a mixture of

40% barium carbonate and 60% strontium carbonate suspended in a 2% solution of nitrocellulose in amyl acetate. The nickel cathode bearing the section was then placed in the electron microscope which was sealed and evacuated. After a good vacuum had been established, the cathode was heated by means of a tungsten filament. The high voltage (5,500 volts) was then turned on and the electron beam was focused on a fluorescent screen and photographed under standardized conditions.

OBSERVATIONS AND DISCUSSION

Examination of the incinerated control and citrated tissues revealed striking changes in the intracellular minerals. The most obvious change involved the glaring white ash which appears to be the chief constituent of the nucleus and cell membrane. Without exception, all of the citrated tissues revealed a pronounced decrease in the amount of this white ash. In many cases, nuclei of cells which in control preparations were extremely rich in white ash showed little or none in the citrated preparations. The nuclei of the epithelial cells in the mucosa of the duodenum (fig. 2) were represented by a ring of white ash, presumably the mineral residue of the nuclear membrane. Figure 2 also demonstrates the low white ash content of the cell membranes as compared with the control material (fig. 1). It is most interesting to note that the white ash removed from the nucleus and cell membrane was not deposited in the cytoplasm but was removed from the cell. Actually, there was very little white ash present in the cytoplasm of citrated cells. An appreciable amount of this ash, however, could be detected in the tissue spaces.

The white ash is generally taken to indicate the presence of calcium and magnesium oxide (Scott, '33). This would seem to demonstrate that incinerated preparations of tissues perfused with sodium citrate contain less calcium and magnesium in the nuclei, cytoplasm and cell membranes than do the controls. That the white ash actually represents calcium and magnesium was confirmed by examination of the specimens studied by means of the electron microscope (fig. 5).

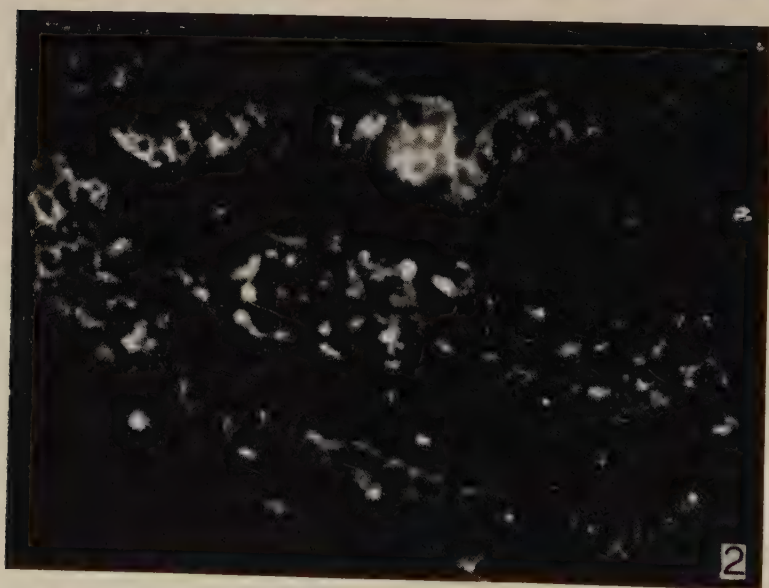
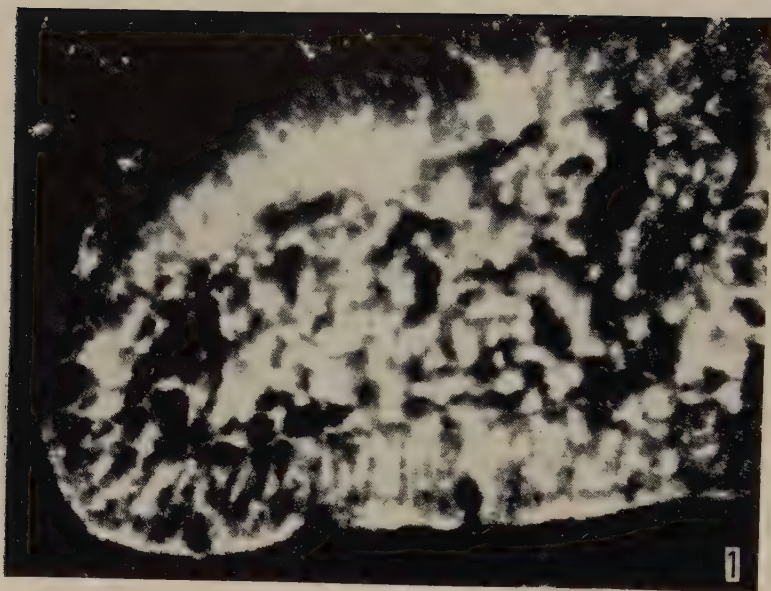


Fig. 1 Photomicrograph of villus of cat duodenum; microincinerated preparation, 4 micra section. Note the large amounts of white ash in the nuclei of the epithelium. Magnification approximately 400.

Fig. 2 Photomicrograph of villus of duodenum of the same cat as shown in figure 1, after perfusion with sodium citrate. Virtually all the calcium has been removed from the nucleus and cytoplasm. Magnification approximately 400.

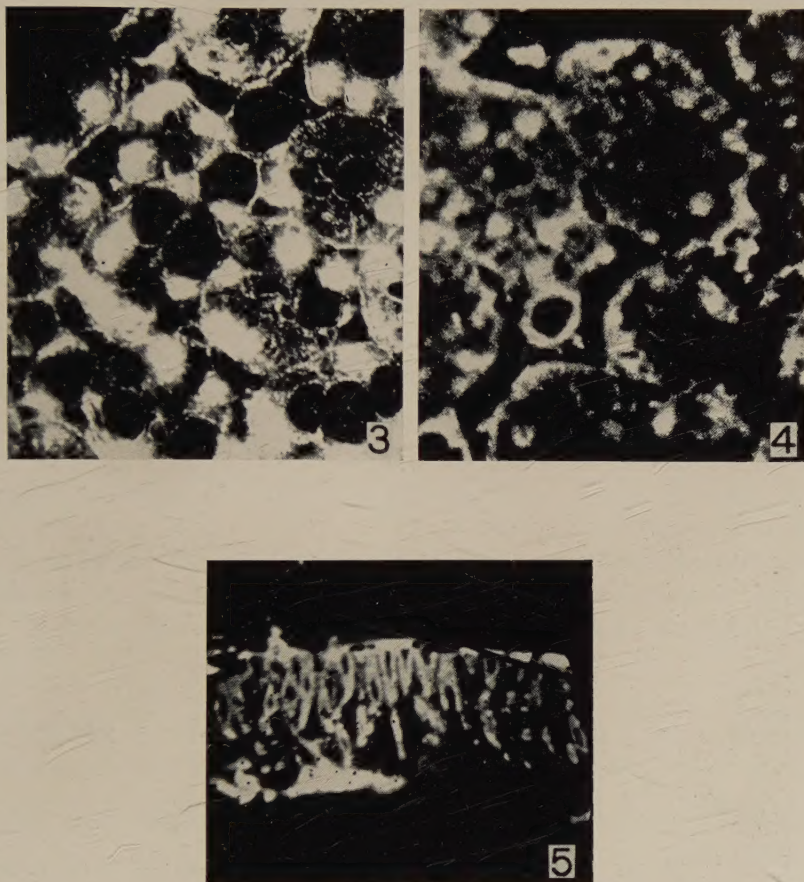


Fig. 3 Photomicrograph of an incinerated control section of cat kidney sectioned at 4 micra. Note the heavy deposits of calcium in the nuclei and membranes. Magnification approximately 400.

Fig. 4 Photomicrograph of an incinerated, citrate perfused section of cat kidney (same cat as shown in fig. 3). Calcium has been removed from the nuclei, cytoplasm and cell membranes. Much of the ash shown in this photomicrograph is the blue ash of sodium. Magnification approximately 400.

Fig. 5 Electron microscope photograph of a control section of cat duodenum sectioned at 4 micra. The epithelium of the mucosa contains large amounts of calcium and magnesium. Magnification approximately 100.

The blue ash in microincinerated preparations is taken to indicate the presence of sodium or potassium or both (Scott, '33). In the present experiments it was found that the blue ash content of the cytoplasm of the various citrated cells as well as the tissue spaces contained an abundance of blue ash. The lumina of blood vessels were filled with this blue ash making it quite evident that it represented the mineral residue of the perfusion fluid. The conclusion was reached from these observations that the oxide of sodium in microincinerated preparations possesses a blue color.

SUMMARY

Cats were perfused with 0.9% sodium citrate which was maintained at body temperature. Control and citrated samples of liver, kidney, duodenum, rectus abdominis muscle and femoral nerve were studied by means of microincineration and electron microscopy.

It was demonstrated that calcium and magnesium are removed from the nucleus, cytoplasm and cell membrane of cells perfused with sodium citrate.

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BOOK REVIEW

PROCEEDINGS THIRD ANNUAL CONFERENCE ON BIOLOGY OF THE SPERMATOOZOA¹ (March 21-22, 1942, New York City).

Published by the National Committee on Maternal Health, Inc.,
Clair E. Folsome, M. D. Executive Director, Ann Arbor, Michigan.

This mimeographed publication is a verbatim record of a conference which was held in New York on March 21-22, 1942. It includes twelve papers and extensive discussion. Thirty of the forty-seven individuals in attendance participated in the discussion. An outstanding feature of the conference was the varied experience of the participants, there being represented such fields as Anatomy, Bacteriology, Biochemistry, Biology, Cytology, Genetics, Immunology, Medicine, Pathology, Physical Chemistry, Physiology and Surgery. Drs. Earl T. Engle (General Chairman), Robert Chambers and Michael Heidelberger served successively as chairmen of the three sessions.

The papers and their authors are as follows: *Cytological studies and spermatozoa*, Dr. Aura Severinghaus; *Comparative and differential fertility*, Dr. L. E. Casida; *Significant developments in the study of human spermatozoa*, Dr. Robert S. Hotchkiss; *Constitutional factors and human fertility*, Dr. George Draper; *The relation of nutrition to spermatogenesis*, Dr. Landrum B. Shettles; *Certain biochemical factors in sperm metabolism*, Dr. Charles A. Zittle; *A comparative study of human sperm metabolism to sperm metabolism of other species*, Dr. John MacLeod; *A new microrespiration apparatus*, Dr. David Bishop; *The chemical composition of normal human semen*, Dr. Charles Higgins; *Some proteins of human seminal fluid*, Drs. Victor Ross and Dan H. Moore; *Prostatic "acid" phosphatase in seminal fluid*, Dr. Alexander B. Gutman; *Is there a serological factor in human infertility?* Dr. Philip Levin.

Much of the material in an analysis of published observations. Yet there are included many hitherto unpublished data and, fortunately, various viewpoints concerning the interpretation of them. Clinical observations and problems receive considerable attention.

¹ Proceedings of the Third Annual Conference on Sperm Biology may be secured without cost upon application to the office of the Committee, 2 East 103 Street, New York, N. Y.

The sex chromosomes of a human sex-intergrade are considered. Careful cytological studies fail to reveal a chromosomal pattern which assists in explaining the cause of the intersexual condition.

The discussion concerning metabolism in spermatozoa deals with the consumption of oxygen and the sources of energy. In experiments in which mammalian spermatozoa are broken into three fragments and these fragments subsequently segregated by centrifuging, the consumption of oxygen is highest in midpieces and tails. The energy for spermatozoa seems to be derived almost entirely from glucose which, although ample in seminal fluid, is thought to be scarce in the epididymis (scarce or absent in fluid from spermatoceles). For spermatozoa within the uterus, glucose may be furnished via the uterine glands during their glycolytic cycle.

It is pointed out that the fluid from human seminal vesicles contains much glucose, protein, inorganic phosphorus and ascorbic acid. The secretion of the prostate has much potassium, calcium and sodium. In the plasma of seminal fluid, there is probably less than 0.02% of albumen and less than 0.04% of nucleoprotein. Whole seminal fluid contains large quantities of an enzyme, "acid" phosphatase, which occurs abundantly in the prostate. An increase of this phosphatase in the blood facilitates the diagnosis of metastasizing prostatic carcinomas.

Among several observations from unfinished experiments, three may be mentioned. The first observation is the impairment of spermatogenesis in response to dietary deficiency of either tryptophane, arginine or lysine. The second concerns an attempt to measure directly, by means of a new microrespirometer, the amount of oxygen used by spermatozoa. It is said that satisfactory determinations are obtainable on 0.02 cc. of material or on as few as 200,000 spermatozoa. The third observation demonstrates that the treatment of spermatozoa with various chemicals prior to insemination fails to modify the sex ratio among offspring.

There are ninety-eight single-spaced pages, nine photomicrographs, one photograph, five text figures and nine tables. Approximately thirty pages are devoted to discussion and four to bibliographies which accompany five of the papers. Typographical errors indicate that more discerning proofreading is needed.

The National Committee is to be congratulated upon having prepared this publication. It has made available to those who could not attend the conference the deliberations of an outstanding group of investigators and thereby fulfilled, in the words of its Director, the function of stimulating, developing and encouraging research in the undeveloped medical aspects of human fertility.

L. J. WELLS,
University of Minnesota.